STUDIES ON NUCLEAR

RNA

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David Salway Holmes

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Abstract

The isolation of giant nuclear RNA (HnRNA) from rat ascites cells is described. By the criteria of sedimentation through sucrose, formaldehyde and dimethyl sulfoxide, it is estimated that the majority of the radioactivity of giant HnRNA after a 30 minute pulse of ^{3}H-uridine is associated with molecules in the range $5\text{-}10 \times 10^{6}$ daltons. In the electron microscope, under denaturing conditions, 84% (mass %) of giant HnRNA has a contour length of 4-9 μ corresponding to a molecular weight of about $5\text{-}10 \times 10^{6}$ daltons.

Giant HnRNA has a "DNA-like" base composition (G+C = 46-54%) and has considerable secondary structure (ca. 60% helix conformation) as judged by its melting profile and reactivity with formaldehyde.

Rat nuclear DNA is characterized by its reassociation profile ($(Na^{+}) = 0.18$ at 62°, $T_m - 23^{o}$) as judged by chromatography on hydroxyapatite. Single-copy DNA (Cot 1/2 observed = 1.5×10^{3}) comprises 65% of the genome and 19% of the genome consists of sequences repeated an average 1,800 times (middle repetitive DNA, Cot 1/2 observed = 1.0). 9% of the genome (highly repetitive DNA) reassociates faster than is measured in these experiments (Cot 1/2 observed $< 2 \times 10^{-2}$).

Middle repetitive and single-copy DNA are isolated and characterized with respect to their reassociation kinetics and melting profiles. They reassociate with kinetics similar to the kinetics describing these components when they are present in total DNA. The reassociated single-copy DNA has a high thermal stability

indicative of fidelity of base pairing; the reassociated middle repetitive DNA has a lower thermal stability which is probably attributable, in part, to base-pair mismatch.

Rat giant nuclear RNA (HnRNA, 5-10 x 10^6 daltons) is hybridized to isolated single copy or middle repetitive DNA ((Na^+) = 0.18 at 62°) HnRNA hybridizes to about 4.5% of the single-copy and 9.4% of the middle repetitive DNA. The T_ms of single-copy and middle repetitive hybrids are 1-2° lower than those of the reassociated single-copy and middle repetitive DNA respectively. The DNA isolated from the single-copy or middle repetitive hybrids reassociates with kinetics similar to the input single-copy or middle repetitive DNA respectively. HnRNA is hybridized to total genomic DNA present in excess. 37% of the HnRNA hybridizes with kinetics (Cot 1/2 = 2.0 x 10^3) similar to single-copy DNA and 12% hybridizes with kinetics (Cot 1/2 = 5.6), a little more slowly than the major reassociating component of middle repetitive DNA.

A chromatin-associated RNA (cRNA) prepared from rat ascites cells hybridizes to about 16% of isolated middle repetitive and 1% of isolated single copy rat DNA. In a hybridization reaction to total DNA, present in excess, at least 50% of the cRNA hybridizes at an average rate similar to the major component of the middle repetitive DNA. These experiments indicate that the majority of cRNA consists of repetitive transcripts. Under conditions which assay essentially only repetitive transcripts cRNA hybridizes to about 4.7% and giant nuclear RNA (HnRNA) hybridizes to about 4.6% of total nuclear rat DNA immobilized on filters. The T_m of cRNA hybrids (73.5°) and HnRNA

hybrids (75.5°) are considerably lower than the T_m of native rat DNA (85.5°). This lowering of T_m is probably attributable, at least in part, to base-pair mismatch. Under the same conditions of hybridization there is some hybridization competition for complementary DNA sites between cRNA and HnRNA, presumably between repetitive transcripts. Due to probable base-pair mismatch it is possible to infer only that there is a similarity between HnRNA and cRNA transcripts and not necessarily an identity.

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CHAPTER 1.

Chromosomal RNA: Its Properties

David S. Holmes, John E. Mayfield, Gernot Sander and James Bonner

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Chromosomal RNA: Its Properties

Abstract. *We describe the properties of a special class of RNA associated with chromatin. We discuss why this RNA should be considered a distinct class of RNA and not an artifactual degradation product of either transfer or ribosomal RNA.*

We have described the preparation and properties of a class of RNA molecules associated with chromosomes, which we have termed chromosomal RNA or cRNA (*1–3*). It has been suggested that cRNA may be an artifact resulting from the degradation of transfer RNA (tRNA) (*4*), or that it may not exist at all (*5, 6*). In this report we describe the known properties of cRNA and show that cRNA from Novikoff ascites in the rat is not detectably contaminated with tRNA, ribosomal RNA (rRNA), or their degradation products. We review the methods available for the isolation of cRNA and discuss whether cRNA should be considered a distinct class of RNA.

Three principal properties identify cRNA. First, it elutes as a symmetrical peak from diethylaminoethyl (DEAE) Sephadex in 7*M* urea at approximately 0.55*M* NaCl (*2, 3, 7–13*) and from DEAE cellulose at 0.38*M* NaCl (*11, 14, 15*). Second, cRNA hybridizes to DNA to a much larger extent than does either tRNA or rRNA (*2, 3, 8, 14*). We have yet to isolate cRNA from any tissue which hybridizes to less than 2 percent of homologous DNA (*2, 3, 8–11, 16*). Further, cRNA from pea buds (*7*) and rat ascites (*8*) hybridizes to the middle-repetitive sequences of homologous DNA. Third, cRNA isolated from a wide variety of organs and organisms contains from 7 to 10 percent dihydropyrimidine (*3, 8–12, 15, 19, 20*).

Both cRNA and tRNA elute from DEAE Sephadex at 0.55*M* NaCl in 7*M* urea. The contamination of cRNA by tRNA is minimized by using isolated chromatin as the starting material. Even if purified tRNA or isolated ribosomal subunits are added to isolated chromatin and the chromatin is processed for cRNA by the method of Dahmus and McConnell (*3*), less than 1 percent of the cRNA peak recovered on DEAE Sephadex is either tRNA or rRNA (*21*). The following observations also demonstrate that cRNA is not related to either tRNA or rRNA.

1) Ascites cRNA can be separated from tRNA on DEAE cellulose (*11, 14, 15*), Sephadex G100 (*8*), or by disc gel electrophoresis (Fig. 1a).

2) If tRNA is subjected to the same Pronase treatment that is used in the isolation of cRNA, no degradation of tRNA to fragments the size of cRNA is detected (Fig. 1, b to e). This result is not in agreement with one report (*4*). The discrepancy is probably due to the quality of Pronase used, that is, to residual ribonuclease activity present in those Pronase samples which do degrade tRNA.

3) Ascites cRNA hybridizes to an estimated 4.9 percent of rat DNA, whereas tRNA hybridizes to about 0.069 percent of rat DNA under the same conditions (Fig. 2, a and b). The relation between base sequences of ascites cRNA and tRNA has been tested by reciprocal competition experiments. At the level of sensitivity of these experiments, no relation could be found between these base sequences (Table 1). Furthermore, cRNA hybridizes exclusively to purified middle-repetitive DNA (*8*) and in a DNA-driven RNA hybridization reaction has a $Cot_{1/2}$

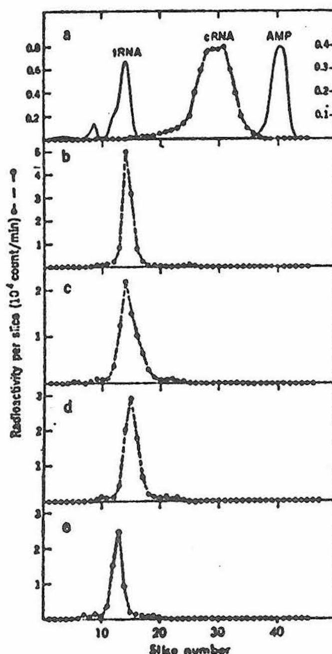

Fig. 1. Separation of tRNA, cRNA, and adenosine monophosphate (AMP) by polyacrylamide gel electrophoresis. Samples were applied in 20 μl of 50 percent glycerol (by volume) to a 6-cm column containing 14 percent acrylamide and 0.1 percent sodium dodecyl sulfate, and 5 ma per gel was applied for 2.5 hours. (a) The sample was [³H]cRNA prepared from rat Novikoff ascites by a method similar to that of Dahmus and McConnell (*3*), yeast tRNA (Sigma), and AMP. Absorbance at 260 nm (scale at right) is given by the solid line. (b) The sample, [³H]tRNA prepared from rat Novikoff ascites as described by Dahmus and McConnell (*3*) and stored at −18°C in 1 m*M* ethylenediaminetetraacetic acid, was applied directly to the gel without prior treatment. (c) The [³H]tRNA in (b) was incubated in 0.01*M* tris(hydroxymethyl)-aminomethane (tris), *p*H 8, at 37°C for 2 hours before electrophoresis. (d) The preparation in (b) was incubated in 0.01*M* tris, *p*H 8, plus Pronase B (Calbiochem), 2 mg/ml, at 37°C for 2 hours before electrophoresis. The Pronase had been first incubated in 0.01*M* tris, *p*H 8, for 90 minutes at 37°C at a concentration of 20 mg/ml. (e) The sample was prepared as in (d) except that Pronase C (Calbiochem), 2 mg/ml, was used in place of Pronase B. The Pronase was first incubated as described in (d).

(product of concentration, in moles of nucleotides per liter and hybridization half-time in seconds) distinct from that of purified tRNA (*8*).

4) Dahmus and McConnell (*3*) reported that ascites tRNA compared to ascites cRNA contains five times more methylated bases and one third the amount of dihydropyrimidine.

5) Jacobson and Bonner (*14*) were unable to find universal nucleotide sequences in cRNA following ribonuclease digestion, whereas tRNA is known to contain such sequences (*22*). In addition they showed (*14*) that despite the high sequence diversity of ascites cRNA the 5′ ends are 90 percent cytosine and the 3′ ends are 99 percent guanine. This result suggests that cRNA is not a random mixture of RNA fragments, although the possibility that this merely reflects the specificity of nucleases cannot be excluded.

6) Additional evidence that ascites cRNA is a distinctive RNA class is derived from in vivo half-life studies (*10*). The decay kinetics of pulse-labeled cRNA follow a linear first-order profile; this suggests that cRNA is a single kinetic component (*10*). In addition, the half-life of cRNA (17 hours) is different from that of any other RNA species known in rat ascites (*10*).

7) Dahmus (*11*) found ascites cRNA to have no detectable amino acid acceptor activity. Purified tRNA, treated with Pronase and chromatographed on DEAE Sephadex by the procedure for the preparation of cRNA, exhibited the same acceptor activity as did untreated tRNA (*11*). This finding contrasts with that of Heyden and Zachau (*4*), who reported the loss of acceptor activity after Pronase treatment of pure tRNA, a result that suggests that their Pronase treatment resulted in RNA degradation.

One of the first methods developed for isolating cRNA was the CsCl (skin) method (*2, 3*). In this method chromatin is dissolved in 4*M* CsCl and centrifuged overnight at 100,000*g*. The proteins aggregate and float to the top of the tube, forming a skin, while the majority of the DNA and the RNA is pelleted. The cRNA is found in the skin and can be released by protease treatment and purified away from other skin RNA's by chromatography on DEAE cellulose.

The CsCl skin method is not a satisfactory procedure for preparing cRNA because (i) other RNA species are apparently trapped adventitiously by the aggregated proteins (*5, 6, 21*), and (ii) treatment of the skin with protease can result in the degradation of RNA if large amounts of endogenous ribonuclease are present in the starting chromatin, or if the protease is contaminated with ribonuclease. Therefore, we wish to draw attention to three additional methods for the preparation of cRNA. In the procedure described by Huang and co-workers (*23*), chromatin is dissolved in 2.5*M* guanidinium chloride and the DNA is pelleted at 105,000*g* for 24 hours. The supernatant, containing proteins and various RNA's, is adjusted to 4*M* CsCl and 2*M* guanidinium chloride and subjected to buoyant density equilibrium centrifugation. Fractions containing cRNA are collected, and the cRNA is further purified by DEAE cellulose chromatography and disc gel electrophoresis. Other methods have been described by Jacobson and Bonner (*14*) and by Mayfield and Bonner (*10*).

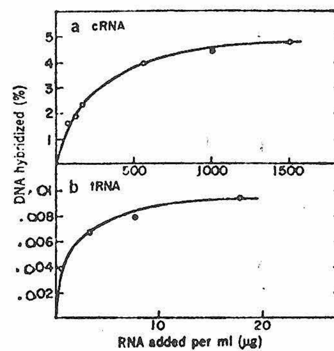

Fig. 2. Hybridization of cRNA and tRNA to homologous DNA. Samples of DNA, cRNA, and tRNA were prepared from rat Novikoff ascites as described by Dahmus and McConnell (*3*). The tRNA was labeled in vitro with [^{3}H]dimethyl sulfate to a specific activity of approximately 180,000 count/min per microgram by a method similar to that of Mayfield and Bonner (*10*), and cRNA was labeled in vivo with [^{32}P]orthophosphate to a specific activity of approximately 4000 count/min per microgram (*3*). Filters containing DNA were prepared by the method of Gillespie and Spiegelman (*17*); 10 μg (a) or 2 μg (b) of denatured DNA with trace amounts of [^{14}C]DNA were used per filter. The hybridization reaction was carried out in 5 × SSC (0.1*M* sodium chloride and 0.15*M* sodium citrate) and 50 percent formamide for 18 hours (a) or 24 hours (b) at 37°C (*18*). At the end of the reaction, unhybridized RNA was removed by incubating the filters in 2 × SSC at 37°C for 30 minutes with pancreatic ribonuclease A, 50 μg/ml, and ribonuclease T1, 50 units/ml (both, Worthington Chemical Co.).

Table 1. Hybridization competition between labeled tRNA and cRNA and unlabeled cRNA, rRNA, and tRNA. Preparation of cRNA, rRNA, and tRNA and of DNA filters was as described by Dahmus and McConnell (*3*). Reaction conditions were described in the legend to Fig. 2 and in (*3*).

Competing unlabeled RNA	Ratio of total RNA to labeled RNA	Labeled hybrid remaining (%)
Labeled cRNA		
None	1	100*
Transfer	3.8	103
Ribosomal	3.8	103
Chromosomal	2.5	76
Chromosomal	3.8	50
Labeled tRNA		
None	1	100†
Transfer	2.1	76
Transfer	5.5	45
Chromosomal	5.1	97

* This is 74 percent of saturation value. † This is 70 percent of saturation value.

The properties of rat ascites cRNA described in this report suggest that it is a distinct class of RNA which is not detectably contaminated with tRNA, rRNA, or their degradation products. The relation of rat ascites cRNA to other nuclear RNA's has been studied (*8*). Principal among the distinguishing features of rat ascites cRNA are its base sequence heterogeneity and its high content of dihydropyrimidine. The data indicate that cRNA preparations from other tissues also contain RNA with these unusual properties, but there is insufficient evidence to eliminate the possibility of contamination by tRNA or degraded rRNA.

Much of the controversy surrounding the existence of cRNA stems from a misunderstanding of its definition. Artman and Roth define cRNA as "the RNA associated with the proteins which float in 4*M* CsCl" (*5*). A similar definition of cRNA is used operationally by Szeszák and Pihl (*6*). The RNA that associates with the CsCl skin is more properly termed "skin RNA," and includes, in addition to cRNA as defined earlier in this report, a heterogeneous collection of RNA molecules that are adventitiously trapped by the proteins that float to the surface of CsCl (*5*). Skin RNA must be further processed on DEAE cellulose to remove these adventitiously trapped RNA's and permit recovery (at least in the case of preparations from rat ascites cells) of purified cRNA. The methods described by Huang and co-workers (*23*) and Jacobson and Bonner (*14*) eliminate the problems inherent in the CsCl skin

method and appear to be the methods of choice for the isolation of purified cRNA.

Much work remains to be done on the characterization of cRNA. We hope that this report will help others to extend these studies and will stimulate further investigations of the cellular function of this RNA.

DAVID S. HOLMES
JOHN E. MAYFIELD
GERNOT SANDER
JAMES BONNER

Division of Biology,
California Institute of Technology,
Pasadena 91109

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16. Unpublished data; cRNA prepared from calf thymus by the method of Mayfield and Bonner (*10*) hybridizes to 2 percent of homologous DNA. The hybridization reaction was carried out as described by Gillespie and Spiegelman (*17*) and McConaughy *et al.* (*18*).
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21. To isolated rat ascites chromatin (*3*) were added [^{3}H]tRNA (3×10^{6} count/min) and [^{32}P]ribosomal subunits (5×10^{7} count/min). Recovered in the CsCl skin (*3*) were 1.3×10^{2} count/min from ^{3}H and 4×10^{3} count/min from ^{32}P, and in the cRNA peak on DEAE Sephadex (*3*) were 96 and 8 count/min from ^{3}H and ^{32}P, respectively, values representing less than 1 percent of the cRNA when the input tRNA and rRNA is normalized to the mass of these species present in ascites cells.
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CHAPTER 2.

Preparation, Molecular Weight, Base Composition and Secondary Structure of Giant Nuclear RNA

ABSTRACT: Previous methods of HnRNA extraction yield material which "disaggregates" into small molecules. This could be the fault of either ribonuclease knicks in the polymers sustained during the extraction procedure or disaggregation into real subunits. The present communication distinguishes between these possibilities by describing an RNA extraction procedure which does not yield subunits when HnRNA is denatured. By the criteria of sedimentation through sucrose, formaldehyde and dimethyl sulfoxide, it is estimated that the majority of the radioactivity of giant HnRNA after a 30 minute pulse of [^{3}H]uridine is associated with molecules in the range 5-10 x 10^6 daltons. In the electron microscope, under denaturing conditions, 84% (mass %) of giant HnRNA has a contour length of 4-9 μ corresponding to a molecular weight of about 5-10 x 10^6. Giant HnRNA has a "DNA-like" base composition (G+C = 46-54%) and has considerable secondary structure (ca. 60% helix conformation) as judged by its melting profile and reactivity with formaldehyde.

Introduction.

The nuclei of mammals contain a class of heterogeneous RNA (HnRNA) that is rapidly labeled, sediments heterogeneously in sucrose gradients, and has a DNA-like base composition (Scherrer *et al.*, 1963; Attardi *et al.*, 1966; Soeiro *et al.*, 1966; Schutz *et al.*, 1968; Soeiro and Darnell, 1970). A portion of HnRNA has a sedimentation coefficient greater than 45S as judged by sedimentation through sucrose. This has been equated with a molecular weight in excess of 4×10^6 daltons, using the available equations to relate molecular weight and sedimentation velocity (Gierer, 1950; Spirin, 1961). However the dangers in using such equations have been pointed out by Gesteland and Boedtker (1964) and Strauss and Sinsheimer (1967). Estimates of the molecular weight of a variety of cellular and viral RNAs have been obtained by other methods such as sedimentation or electrophoresis in denaturing solvents (Boedtker, 1968; Fenwick, 1968; Strauss *et al.*, 1968; Staynov *et al.*, 1972), light scattering (Gesteland and Boedtker, 1964), viscosity (Mitra *et al.*, 1963) and electron microscopy (Granboulan and Scherrer, 1969; Robberson *et al.*, 1971). However there are few reports in the literature in which such techniques have been applied to HnRNA. Granboulan and Scherrer (1969) describe a class of HnRNA molecules with a molecular weight in the range $5\text{-}10 \times 10^6$ daltons as judged by visualization in the electron microscope under partially denaturing conditions. On the other hand, Mayo and de Kloet (1971) using formaldehyde sucrose gradients, and Scott and Kuhns (1972) using electrophoresis in the presence of DMSO[1] present

evidence that giant HnRNA "disaggregates" into smaller molecules presumably because denaturation reveals hidden nicks in the RNA.

This paper describes a method for the isolation of giant HnRNA from rat ascites cells which does not disaggregate under denaturing conditions. Such RNA has low GC content and is heterogeneous in size with a molecular weight in the range $5\text{-}10 \times 10^6$ daltons as judged by sedimentation and electron microscopy under denaturing conditions. The helix-coil transition and reactivity with formaldehyde indicate that this giant HnRNA has considerable secondary structure *in vitro*.

Methods

Isolation of Nuclei. Male albino Sprague-Dawley rats (Berkeley Pacific Laboratories) were injected 6 days prior to harvest with 0.5 ml of Novikoff ascites fluid per rat. For the preparation of [^{3}H]uridine-labeled HnRNA, rats were given an intraperitoneal injection of [^{3}H]uridine (New England Nuclear Corp., 180 Ci/M) prior to harvesting the fluid. Details of the amount of isotope and labeling time appear in the descriptions of individual experiments. Generally one rat yielded approximately 20 ml of fluid with a concentration of $1\text{-}5 \times 10^7$ ascites cells/ml.

Except where stated all steps were carried out at 4°. Ascites fluid was centrifuged at 1000*g* for 30 sec, packed cells were suspended with the aid of a spatula in about 10 volumes of deionized water and then centrifuged at 1500*g* for 5 min. This yields

a pellet of crude nuclei as judged by phase contrast microscopy.

Isolation of Total Nuclear RNA. (I.) The pellet of crude nuclei was lysed at room temperature in approximately 25 volumes of lysis mixture (2% SDS [Matheson, Coleman and Bell, recrystallized from ethanol], 7 M urea [Schwarz-Mann Ultra Pure], 0.35 M NaCl, 1 mM EDTA, 0.01 M Tris [pH 8]). Reagent grade urea but not Mann "Ultra Pure" urea, degrades RNA in solution (Sedat et al., 1969). An equal volume of phenol mixture (see Miscellaneous Methods) was added and the mixture shaken at room temperature for 15 min. The phases were separated by low speed centrifugation and the phenol phase and interface reextracted with 1/2 vol. of lysis mixture. The combined aqueous phase was reextracted with 1/2 vol. of phenol mixture and the final aqueous phase (II) precipitated by the addition of 25% sodium acetate (pH 6) to 2% followed by two volumes of cold 95% ethanol. Following storage at -18° for 2-4 hr the DNA was spooled out, the flocculent precipitate pelleted at 12000g for 10 min, and the pellet washed once with cold 95% ethanol. The DNA and the pellet were partially resuspended in TNM (0.01 M Tris HCl (pH 8), 0.01 M NaCl, 0.01 M $MgCl_2$) with the aid of a spatula, DNase I (Worthington Co., repurified, see below) was added to a final concentration of 100 µg/ml and the mixture incubated at 37° for 3 min using constant agitation with a spatula. The reaction was stopped by plunging in ice, followed by the addition of 25% SDS to 2% and 0.1 M EDTA to 1 mM. An equal volume of phenol mixture was added and the mixture extracted at 4° as described above. The combined aqueous phases (III) were precipitated as described above.

This precipitate contains the majority (66%) of the nuclear RNA.

Sepharose B Chromatography. Total nuclear RNA was resuspended in 2 mM phosphate buffer (pH 6.8)-1 mM EDTA and subjected to chromatography on Sepharose 2B (Pharmacia; column 2.5 x 45 cm), in the same buffer at a flow rate of approximately 15 ml/hr (Baltimore, 1968; Oberg and Philipson, 1969; Clements and Martin, 1971). 25 μl aliquots of each fraction were counted in Aquasol and dilutions were made for absorbance readings. Up to 10 mg of RNA in 4 ml of buffer have successfully been fractionated as described. Fractions corresponding to the excluded volume of the column were pooled and precipitated as described above (IV-V).

Sucrose Gradients. (1) Non-denaturing. Preformed linear 5-20% sucrose (Schwarz-Mann Ultra Pure) gradients in 0.1 M NaCl-1 mM EDTA-0.01 M sodium acetate (pH 6)-0.2% SDS, were run at 39,000 rev/min at 18° in the Spinco SW-39 rotor. 20-100 μg of RNA were layered on the gradients in 100 μl of the same buffer as the sucrose gradient. In some instances the RNA was heated at 80° in 1 mM EDTA (pH 8) for 2 min and cooled to about 20° in dry ice-ethanol prior to loading. Using a first order rate constant of 5×10^{-8} sec^{-1} (Eigner et al., 1961), and the equation of Spirin (1961) to relate sedimentation coefficient to molecular weight, it is estimated that the sedimentation coefficient of RNA should fall by about 5% after such heat treatment. The gradients were dripped onto Whatman 3 mM filter paper; washed in succession with two changes of cold 10% trichloroacetic acid and two changes of

95% ethanol. The filters were air-dried, vacuum oven-dried, and counted in a toluene-based scintillation cocktail. (2) Denaturing formaldehyde gradients. RNA was sedimented in formaldehyde-containing sucrose gradients as described by Fenwick (1968). The gradients were as described above but without SDS and including 6% formaldehyde (Mallinckrodt, reagent grade). The samples were heated at 55° for 5 min in the same buffer prior to loading.

Dimethyl Sulfoxide Gradients. Centrifugation of RNA in a linear dimethyl sulfoxide (DMSO) gradient was carried out essentially by the method of Strauss *et al.* (1968) and Sedat *et al.* (1969), with the following modification: 20-50 µg of RNA was resuspended in 100 µl dimethyl sulfoxide (Matheson, Coleman and Bell, Spectrograde quality) and heated at 60° for 2 min prior to loading on the gradient. The gradients were assayed for radioactivity as described above.

Preparation of Ribosomal RNA. Crude ribosomes were isolated from a cytoplasmic extract of rat ascites cells by the method of Moldave and Skogerson (1967). RNA was isolated from the ribosomes by phenol extraction and purified by centrifugation on a 5-20% linear sucrose gradient for 16 hr at 25000 rev/min at 18° in the Spinco SW-25.2 rotor as described above. Fractions corresponding to the peaks of 28S and 18S rRNA were precipitated and rerun separately in 5-20% linear sucrose gradient as described above.

E. coli rRNA was a gift from Lloyd Smith and wheat germ rRNA was purchased from Calbiochem.

Preparation of Ribosomal RNA Precursor. Rats were labeled for 1 hr prior to the harvest of ascites cells by intraperitoneal injection of 1 mC [^{3}H]methylmethionine/rat (Schwarz). Crude nuclei were prepared from rat ascites cells as described. Nucleoli were prepared by the method of Jeanteur et al. (1968). Nucleic acids were extracted from the nucleoli by phenol extraction as described previously. Following precipitation the nucleic acids were suspended in TNM and subjected to DNase I treatment, followed by phenol extraction and precipitation as described above. The purified nucleolar RNA was layered on a 5-20% linear sucrose gradient as described above. Sedimentation was for 130 min at 39000 rev/min at 18° in the Spinco SW-39 rotor. The tubes corresponding to the peak 45S RNA were pooled, precipitated and rerun on a 5-20% linear sucrose gradient as described above.

Purification of DNase. Electrophoretically pure DNase I was purchased from Worthington Biochemical Co. Different lots varied considerably in their ability to degrade HnRNA under standard conditions and it was found necessary to routinely repurify the DNase. 1 mg DNase I was dissolved in 1 ml of 0.3 M NH_4-acetate, pH 5.8, and chromatographed on Sephadex G50 (fine; column 20 x 3 cm). Fractions which corresponded to the peak activity were pooled and dialyzed against 0.01 M NH_4-acetate, pH 5.8. The DNase was used immediately.

Determination of Sedimentation Coefficients. Estimation of RNA sedimentation coefficients in formaldehyde was performed by band sedimentation in the Model E analytical ultracentrifuge

essentially by the method of Boedtker (1968) with the following modification: RNA was heated at 55° for 5 min in 6% formaldehyde + 0.01 M monosodium and 0.09 M disodium phosphate prior to loading onto 6% formaldehyde in the same buffer made with D_2O. We thank Robert Watson for running the Model E.

Electron Microscopy. RNA was prepared for electron microscopy by the method of Robberson *et al.* (1971) with the exception that Mann Ultra-Pure urea was used in place of reagent grade urea; 45S rRNA precursor was used as a standard. We thank Douglas Ridder for his expert preparations of RNA for the electron microscope.

Nucleotide Analysis. [^{32}P]labeled RNA was prepared by intraperitoneal injection of 2 mC [^{32}P] 1 hr prior to harvest of ascites fluid. RNA was incubated at 37° for 18 hr in 0.3 N KOH. At the end of incubation it was neutralized with 3 N $HClO_4$, allowed to flocculate at 4° for 30 min, and the insoluble salt pelleted by low speed centrifugation. The supernatant was desalted by passage through activated charcoal as described by Sedat (1971). Ribonucleotides were separated on a Picker Nuclear LCS 100 automated nucleotide analyzer. The 2'-3' ribonucleotides standards were purchased from Calbiochem.

Thermal Denaturation of RNA. Melting of RNA was carried out in a Gilford automatic recording spectrophotometer equipped with a thermostatically controlled water bath. Correction was made for the thermal expansion of water.

Analytical Methods. Protein was assayed by the method of Lowry et al. (1951) using BSA as a standard. RNA was determined by the orcinol reaction of Dische and Schwartz (1937) using purified yeast tRNA as a standard. DNA was determined by the diphenylamine reaction described by Burton (1956), using rat ascites DNA as a standard.

Miscellaneous Methods. (1) Phenol distillation. One day before use, phenol (Fisher) was redistilled under N_2, immediately diluted with an equal volume of chloroform (Matheson, Coleman and Bell, Spectro Grade quality), made 1% (v/v) isoamyl alcohol and 0.1% (w/v) 8-hydroxy-quinoline, saturated with water, and stored under pressure in an N_2 atmosphere at -18°. (2) Water for making all solutions was distilled, deionized, and glass distilled. (3) All glassware and metal utensils were heated at 180° for at least 5 hr. (4) All plastic-ware was stored in 0.1% SDS + 0.1% EDTA and washed in succession in water, methanol, water before use. (5) Gloves were used for handling all equipment.

Results

Yield. The yield of acid precipitable counts at various steps in the isolation of nuclear RNA is given in Table I.

Sepharose 2B Chromatography. Figure 1 shows the pattern of separation of radioactivity associated with total nuclear RNA on a column of Sepharose 2B. The majority of the radioactivity (66%) after a 30 min pulse of [^{3}H]uridine, but very little of the mass, is eluted in the excluded volume together with a marker of 45S rRNA precursor. There are some residual DNA oligonucleotides in the included volume of the column even after the DNase digestion

and it is therefore not possible to resolve other classes of RNA on the basis of absorbance.

Sucrose Density Gradient Sedimentation. Figure 2a shows the sedimentation pattern of Sepharose 2B excluded RNA on a linear 5-20% sucrose gradient. The majority of the radioactivity sediments faster than an *E. coli* rRNA marker, and is polydisperse with some indication of peaks at 45S and 70S. The inclusion of EDTA and SDS in the gradients makes it unlikely that these high sedimentation coefficients are the result of aggregation of the RNA due to heavy metal ions or contamination with proteins. No DNA (detection limit 1%) could be detected in the RNA. Thermal denaturation of the RNA prior to centrifugation results in a very slight decrease in the sedimentation coefficients which can probably be attributed to thermal scission of phosphodiester bonds during heating (see Methods).

Figure 2b shows the sedimentation profile of isolated 70S and 45S RNA in a second cycle of sucrose density gradient centrifugation. It is clear that the RNA maintains its integrity on a second passage through sucrose. Using values of 70S and 45S for the modal sedimentation coefficients of the RNA we obtain molecular weight estimates of 1.26×10^7 daltons and 4.77×10^6 daltons, respectively, using the equation:

$$M = 1100\ S^{2.2}$$ (Gierer, 1950);

or 1.18×10^7 daltons and 4.6×10^6 daltons, respectively, using the equation:

$M = 1550\ S^{2.1}$ (Spirin, 1961).

However, the reliability of molecular weight estimates from sedimentation coefficients in sucrose has been criticized due to undefined conformational differences in RNA molecules (Gesteland and Boedtker, 1964; Strauss and Sinsheimer, 1967). We have therefore examined the sedimentation pattern of excluded RNA under conditions which minimize differences in conformation by elimination of base-pairing.

Sucrose Density Gradient Centrifugation in the Presence of Formaldehyde. Low concentrations of formaldehyde efficiently denature RNA (Boedtker, 1967; Fenwick, 1968). As shown in Figure 3, excluded RNA treated with formaldehyde at 55° for 5 min and run on a sucrose gradient in the presence of 6% formaldehyde (Fenwick, 1968) maintains a position relative to marker RNAs which is comparable to that found in a non-denaturing sucrose gradient. This result differs from the report of Mayo and de Kloet (1971) who showed that HnRNA isolated from Ehrlich ascites cells "disaggregated" into smaller molecules after treatment with formaldehyde. During the initial stages of this investigation our preparations of giant HnRNA also had a tendency to "disaggregate" presumably because the denaturing action of the formaldehyde revealed breaks in the HnRNA which were not apparent on non-denaturing sucrose gradients. However, the cause of this breakage was traced to residual ribonuclease activity in the commercial DNase used.

Molecular Weight in Formaldehyde. Sedimentation coefficients of RNA in formaldehyde were obtained in the Model E analytical ultracentrifuge essentially by the method of Boedtker (1968) using *E. coli* 23S and 16S rRNA and rat ascites 45S, 28S and 18S rRNA as standards. RNA eluted in the void volume of Sepharose 2B sedimented as two broad peaks with some tailing on the low molecular weight side. The modal sedimentation coefficients of the peaks were 31 $\pm$ 2.0 and 20.5 $\pm$ 1.5 which correspond to molecular weights of approximately 1.1×10^7 and 3.6×10^6 daltons respectively (Boedtker, 1968). A decrease of about 1% in the $S_{20,w}$ values is expected from thermal degradation of the RNA during its preparation for centrifugation, which is well within the experimental error of $\pm$ 7% for RNA from different preparations.

However, two lines of evidence suggest that the reaction of RNA with formaldehyde could yield spurious estimates of molecular weight. It is known that formaldehyde has no effect on single-stranded stacking interactions (Stevens and Rosenfeld, 1966) which have been shown to have an effect on the radius of gyration of synthetic RNA (Inners and Felsenfeld, 1970). Also, formaldehyde may alter the radius of gyration by formation of methylene bridges (Feldman, 1967).

Sedimentation On a Dimethylsulfoxide Gradient. Under defined conditions dimethylsulfoxide   is known to completely denature a number of single-stranded RNAs, to inhibit ribonuclease, and to have no effect on the biological activity of viral RNA (Strauss

et al., 1968; Sedat *et al.*, 1969). Figure 4 is a standard curve of RNA species of known molecular weight sedimented on DMSO gradients essentially by the method of Sedat *et al.* (1969). Log molecular weight has a linear relationship with sedimentation distance in agreement with Sedat *et al.* (1969) and McGuire *et al.* (1972).

Figure 5a shows the DMSO gradient centrifugation pattern of radioactive RNA eluted in the excluded volume of Sepharose 2B. The majority of the radioactivity sediments more rapidly than *E. coli* rRNA corresponding to molecular weights between 1-10 x 10^6 daltons.

RNA from fractions 11-18 in Figure 5a was pooled, precipitated and rerun on a DMSO gradient (Figure 5b). The RNA maintains its integrity on a second passage through DMSO and has molecular weights between 5-10 x 10^6 daltons, as judged by the radioactivity profile.

It was found necessary to heat both giant HnRNA and 45S rRNA prior to centrifugation in DMSO. If the heating was omitted, 90-95% of the RNA pelleted. Although heat treatment was not necessary to solubilize *E. coli* 23S and 16S rRNA in DMSO, if such RNA was added to the HnRNA sample prior to centrifugation and the heating omitted, then the rRNA also pelleted with the HnRNA. Control experiments in which *E. coli* 23S and 16S rRNA were heated as described showed no detectable decrease in molecular weight of these species. Simmons and Strauss (1972) have observed a similar phenomenon using Sindbis 49S RNA.

We can offer no definite reason for the apparent insolubility of the RNA prior to heat treatment. Although 45S rRNA sediments in DMSO in a position consistent with its molecular weight, it is possible that large RNAs (>28S rRNA) are close to precipitation in DMSO under the conditions used, and, therefore, our estimate of the molecular weight of giant HnRNA in DMSO should be viewed with some reservation.

Electron Microscopy. Figure 6 shows electron micrographs of giant HnRNA purified through DMSO. Figure 7a shows a frequency distribution of the lengths, and Figure 7b shows the mass distribution of this RNA. Table II lists the parameters that describe these distributions. Giant HnRNA is heterodisperse with 84% of the mass of the RNA or 63% of the molecules having a contour length of 4-9 μ. 45S rRNA purified through DMSO has a more uniform distribution with 83% of the mass of the RNA or 53% of the molecules with a contour length between 3-4 μ. Using a value of 1.29×10^6 daltons/1 μ derived from the study of 18S rRNA (Robberson et al., 1971) 63% of the giant HnRNA has a molecular weight in the range $5\text{-}11 \times 10^6$ daltons with a weight average molecular weight of about 7.8×10^6 daltons in reasonable agreement with the results of Granboulan and Scherrer (1969). Similarly 53% of the 45S rRNA has a molecular weight of about $3.9\text{-}5.2 \times 10^6$ daltons with a weight average molecular weight of about 4.3×10^6 daltons. This is consistent with the estimate of 4.3×10^6 daltons derived from the sedimentation velocity of 45S rRNA in DMSO presented above.

Two possible sources of error in the estimate of the length of 45S RNA and giant HnRNA in the electron microscope should be mentioned. First, a number of molecules of HnRNA could not be measured due to ambiguous contour lengths resulting from a lack of complete denaturation. Examples of these are shown in Figure 6d. Some of the molecules whose lengths were measured had small bushes, and regions of apparent secondary structure, which were assumed to be and were measured as duplex regions. Second, our data are uncorrected for the effect of base composition on the contour length of the RNA because the relationship is not well documented (Robberson et al., 1971).

Base Composition. Table III shows the base composition of both the giant HnRNA and 45S RNA isolated from either a sucrose or a DMSO gradient. The giant HnRNA has a "DNA-like" base composition (rat DNA = 41% GC) and relatively high U content. These results are consistent with the findings of others (Attardi et al., 1966; Scherrer et al., 1966; Soeiro et al., 1966; Soeiro et al., 1968). The slightly higher GC content of sucrose purified giant HnRNA compared to that isolated on a DMSO gradient indicates that it is probably contaminated with 45S rRNA precursor. The high GC content of 45S RNA supports the view that a considerable proportion of it is 45S rRNA precursor (rat ascites 45S rRNA precursor = 68% GC).

Assuming that 20% of HnRNA molecules have 1 poly A segment of about 200 A's per molecule (Greenberg and Perry, 1972) then

the contribution of poly A to overall A content of HnRNA (about 4000 A's per HnRNA molecule) is negligible.

Secondary Structure. The relative absorbance-temperature profile of sucrose purified giant HnRNA in sodium phosphate buffer (pH 6.8) is shown in Figure 8a; wheat germ rRNA (51% GC) is included in the figure for comparison. The relative increase in absorbance at 260 mμ of giant HnRNA is 24% (20% hypochromicity) and the T_m is 56.5°. Using an estimate of 29.5% hypochromicity for the helix-coil transition of a completely helical RNA (GC = 50%), obtained by adding the hypochromic contributions of poly (A + I) and polydeoxy (G + C) (Doty, 1962), it is estimated that 68% of giant HnRNA is in a helix conformation (uncorrected for the contribution of single-stranded stacked bases).

A Van't Hoff plot of the temperature dependence of absorbance at 260 mμ of giant HnRNA and wheat germ rRNA is shown in Figure 8b. Assuming a single equilibrium constant, ΔH for the change is 25 Kcal/mole of linkages for both types of RNA, which is comparable to the ΔH = 20 Kcal/mole of linkage found for calf liver microsomal RNA (Hall and Doty, 1959).

The reaction of formaldehyde with the free amino groups of bases is a convenient measure of the extent of hydrogen bonding in RNA (Hall and Doty, 1959; Haselkorn and Doty, 1961; Mitra *et al.*, 1963). The reaction can be followed at 275 mμ in 0.12 M sodium phosphate buffer (pH 6.8) at 25°. The extent of denaturation can be monitored at 245 mμ. Under these conditions there is very

little denaturation of HnRNA. The reaction is pseudo first order with respect to formation of methylol adducts (Penniston and Doty, 1963). Figure 6c shows a plot of the extent of reaction of formaldehyde with free mononucleotides and sucrose purified giant HnRNA. The pseudo first order rate constants derived from the slopes are 2.25×10^{-2} min^{-1} for free mononucleotides and 0.98×10^{-2} min^{-1} for giant HnRNA. Assuming that formaldehyde reacts at the same rate and to the same extent with available bases in giant HnRNA as it does with free bases then a comparison of the rate constants indicates that about 57% of the bases of giant HnRNA are not available for reaction under the conditions used.

Discussion

The preparation of HnRNA presents a number of technical problems which, although probably not unique, are comparatively more serious than in the preparation of ribosomal or transfer RNA. These are: (1) the tendency of HnRNA to aggregate with proteins and escape into the interface during phenol extraction (Parish and Kirby, 1966; Kidson *et al.*, 1964); (2) its susceptibility to nucleolytic digestion during isolation; and (3) the lack of an all-encompassing simple separation procedure due to the range of responses of HnRNA to the usual separation techniques, for example, its polydispersity in a centrifugal field.

The tendency of HnRNA to be found complexed with protein during isolation may reflect its *in vivo* association with protein in the

form of ribonucleprotein particles (Georgiev and Samarina, 1972), or it may be just the tendency of a large polyanion to electrostatically bind some of the more basic proteins (Girard and Baltimore, 1966). We have attempted to overcome this problem by including 7 M urea in the mixture used to lyse the nuclei and in all subsequent phenol extractions. The observation that 93% of the radioactivity after a 30 minute pulse of [^{3}H]uridine is associated with the aqueous phase in the first phenol extraction is a measure of the success of this procedure. One of the most frequently used techniques for the isolation of HnRNA from a nuclear lysate involves phenol extraction at elevated temperatures (Georgiev _et al._, 1963). In our hands a phenol extraction and reextraction at 60° results in partially degraded HnRNA as judged by sedimentation on DMSO, although the RNA still appears to be "undegraded" on a non-denaturing sucrose gradient (unpublished data). Recently a procedure using "chaotrophic" agents such as the lithium salt of trichloroacetic acid to solubilize pulse-labeled RNA during phenol extraction has been published (Scott and Kuhns, 1972). The authors report good yields of RNA, although there is a tendency for the pulse-labeled RNA to "disaggregate" under denaturing conditions.

The use of urea throughout the extraction of HnRNA yields molecules which do not "disaggregate" in denaturing conditions; presumably by reducing ribonuclease activity, and by four criteria, sedimentation through sucrose, formaldehyde, and DMSO and by

electron microscopy we are able to isolate a population of heterogeneous rapidly-labeled nuclear RNA molecules of very high molecular weight.

A variety of methods for following the processing of HnRNA have been developed. For example, the selection of a system where there is little rRNA synthesis (Attardi _et al._, 1966) or where rRNA synthesis has been reduced by drugs (Scherrer _et al._, 1966); by separation of particular classes of HnRNA, e.g., that which is tenaciously bound on columns of methylated albumin kieselguhr (Billing and Barbiroli, 1970), or that which sediments as giant HnRNA on sucrose gradients (Attardi _et al._, 1966; Warner _et al._, 1966); or by following changes in the base ratio of nuclear RNA. We chose to use a separation procedure based on size because it yielded preparative amounts of HnRNA which were, by the criterion of size and base composition comparatively free from 45S rRNA precursor. Our procedure is essentially an adaptation of the frequently used separation of giant HnRNA by sucrose density gradient centrifugation. The novel aspects are the use of urea in the extraction of RNA from nuclei and the introduction of chromatography of whole nuclear RNA on Sepharose 2B. The Sepharose 2B column chromatography represents a convenient way of isolating comparatively large quantities of $\geqslant$ 45S RNA from bulk nuclear RNA without recourse to a considerable number of sucrose gradients. This then allows the separation of giant HnRNA from 45S rRNA precursor in preparative quantities by

centrifugation. For this step both non-denaturing sucrose gradients and DMSO gradients were used. The former allows more RNA to be handled but yields an RNA which is slightly degraded and which contains a small amount of 45S RNA contamination. The latter yields an RNA which is almost completely undegraded and has a very low (46%) GC content.

Giant HnRNA, isolated from a sucrose gradient, has considerable secondary structure in solution as judged by its melting profile and reaction with formaldehyde. This may be related to the finding of Georgiev _et al_. (1971) and Jelinek and Darnell (1972) who have demonstrated the existence of ribonuclease-stable regions in HnRNA which are presumably base-paired. It is not clear at present, whether the observed secondary structure _in vitro_ has any relation to that _in vivo_, but it is tempting to speculate on the relationship of HnRNA secondary structure to its subsequent processing.

Our conclusion is that it is possible to isolate giant HnRNA which, by a variety of criteria, consists of molecules with a molecular weight in the range 5-10 x 10^6 daltons and that do not disaggregate under denaturing conditions. This RNA has a "DNA-like" base composition and considerable secondary structure in solution. Subsequent papers will examine the nature and distribution of the hybridizable sequences present in giant HnRNA.

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TABLE I. Yield of Acid-Precipitable Counts at Various Steps in the Isolation of Giant HnRNA.

	Acid-Precipitable Counts of $[^3H]$uridine	% Counts Remaining
I.[a] Crude nuclear lysate	8×10^7	100
II. Combined aqueous phases after first phenol extraction	7.4×10^7	93
III. Combined aqueous phases after DNase step and second phenol extraction	5.3×10^7	66
IV. Included volume of Sepharose 2B column (<45S RNA)	1.8×10^7	22
V. Excluded volume of Sepharose 2B column (≥45S RNA)	3.4×10^7	42

[a] Roman numerals refer to steps described in Methods.

An aliquot from each step was precipitated with cold 10% trichloroacetic acid (TCA). The precipitate was collected onto a nitrocellulose filter, washed with cold 10% TCA followed by 60% ethanol. The filter was dried and dissolved in 1 ml ethyl acetate and counted in a toluene-based scintillation cocktail.

TABLE II. Length Measurements and Distribution Parameters for RNA. The Data Presented in this Table were Obtained from Figure 6.

	RNA	
	45S	giant Hn
Number average length L_N (μ)	2.91	4.68
Weight average length L_W (μ)	3.36	6.03
Modal length (μ)	3.5	6.0
Tn (μ)[a]	1.2	2.48
Tw (μ)[b]	0.87	1.96
Tw/Lw	0.26	0.32
Molecular weight[c] (daltons) x 10^{-6}	4.33	7.78

[a]Standard deviation based on number-average length.

[b]Standard deviation based on weight-average length.

[c]Molecular weight of the weight average length where1 μ corresponds to 1.29 x 10^6 daltons based on an examination of rRNA under similar conditions (Robberson et al., 1971).

TABLE III. Base Composition of Various Classes of RNA

Species of RNA	Mole %					% ^{32}P				
	C	U	A	G	GC	C	U	A	G	GC
Giant HnRNA[a]	26.0	24.4	21.5	28.1	54.1	23.8	25.6	22.6	27.0	51.8
Giant HnRNA[b]	24.2	30.1	24.1	21.3	45.5	26.1	29.3	24.1	20.5	46.6
45S RNA[c]	28.1	18.4	18.6	34.9	63.0					
Precursor[d]	33.1	17.2	14.7	35.1	68.2					

[a]Sucrose purified giant HnRNA (fraction 0-18 Figure 2b).

[b]DMSO purified giant HnRNA (fractions 11-18 Figure 5a).

[c]Sucrose purified 45S RNA (fractions 19-28 Figure 2b).

[d]45S rRNA precursor from purified rat nucleoli.

FIGURE 1: Chromatography of total nuclear RNA on Sepharose 2B. ●—●, A_{260}; ●---●, ^3H radioactivity. Rat ascites cells were labeled for 0.5 hr with 2 mC [^3H]uridine as described in Methods. The positions of elution of 45S rRNA precursor, 28S + 18S rRNA and tRNA standards are shown. The sample was applied in, and the column eluted with, 2 mM phosphate buffer pH 6.8 + 1 mM EDTA. The column dimensions were 2.5 x 45 cm. The flow rate was about 15 ml/hr and the fraction volume was about 3.6 ml (125 drops).

FIGURE 2: Sedimentation of RNA in a linear 5-20% sucrose gradient containing 0.1 M NaCl-1 mM EDTA-0.01 M Na acetate (pH 6)-0.2% SDS. The sample was applied in 100 µl of the same buffer. The gradients were centrifuged at 39,000 rev/min at 18° in the Spinco SW-30 rotor for 90 min. (a) The radioactive profiles of three gradients have been superimposed in this figure, using *E. coli* 23S + 16S rRNA as a marker. ^3H excluded RNA (fractions 10-15, Sepharose 2B, Figure 1) before ●—● and after ▲---▲ heat treatment. Δ---Δ, ^3H 45S rRNA precursor from purified rat nucleoli. ●---●, ^{14}C *E. coli* 23S + 18S rRNA. Fraction 0 corresponds to RNA which has pelleted. (b) ●—●, fractions 0-18 and ●---● , fractions 19-28 from a gradient similar to that depicted in Figure 2a. Two separate sucrose gradients have been superimposed in this figure and the position of sedimentation of ^3H 45S rRNA precursor from purified rat nucleoli and ^{14}C *E. coli* 23S + 16S rRNA have been indicated by ticks.

FIGURE 3: Sedimentation of RNA in a linear 5-20% sucrose gradient containing 0.1 M NaCl-1 mM EDTA-0.01 M Na acetate (pH 6)-6% formaldehyde. The RNA was heated at 55° for 5 min in 100 µl of the same buffer prior to loading. The gradients were centrifuged at 39,000 rev/min at 18° in the Spinco SW-39 rotor for 200 min. ●——●, ^3H excluded RNA (fractions 10-15 Sepharose 2B, Figure 1); Δ---Δ, ^3H 45S rRNA precursor from purified rat nucleoli; ●---●, ^{14}C *E. coli* 23S + 16S rRNA. The radioactive profiles from two gradients have been superimposed in this figure.

FIGURE 4: Dependence of log molecular weight on sedimentation distance for various RNA classes on DMSO. The DMSO gradients were run as described in Methods. The classes of RNA are tabulated below. The estimate of the molecular weight of the RNA species and its standard deviation refers to the appropriate peak fraction only.

Species of RNA	Molecular weight of ascites RNA (daltons)	No. of expts.	Mol. wt. (daltons) from the literature	Reference
E. coli 16S rRNA			0.55×10^6	Stanley & Bock (1965)
E. coli 23S rRNA			1.07×10^6	Stanley & Bock (1965)
Rat 18S rRNA	$0.69 \times 10^6 \pm 0.03 \times 10^6$	2	0.70×10^6	Loening (1968)
Rat 28S rRNA	$1.74 \times 10^6 \pm 0.08 \times 10^6$	2	1.75×10^6	Loening (1968)
Rat 45S rRNA precursor	$4.32 \times 10^6 \pm 0.26 \times 10^6$	3	4.4×10^6	McConkey & Hopkins (1969)

FIGURE 5: Sedimentation of RNA on DMSO gradients. The gradients were prepared as described in Methods. (a) Sedimentation of ^{3}H excluded RNA (fractions 10-15 Sepharose 2B, Figure 1) ●——●, and E. coli 23S + 16S rRNA ●---●. Centrifugation was for 8 hr at 27° at 65,000 rev/min in the Spinco SW-65 rotor. (b) Sedimentation of giant HnRNA (fractions 11-18 Figure 5a) ●——●, and E. coli 23S + 16S rRNA ●---●. Centrifugation was for 10 hr as described above.

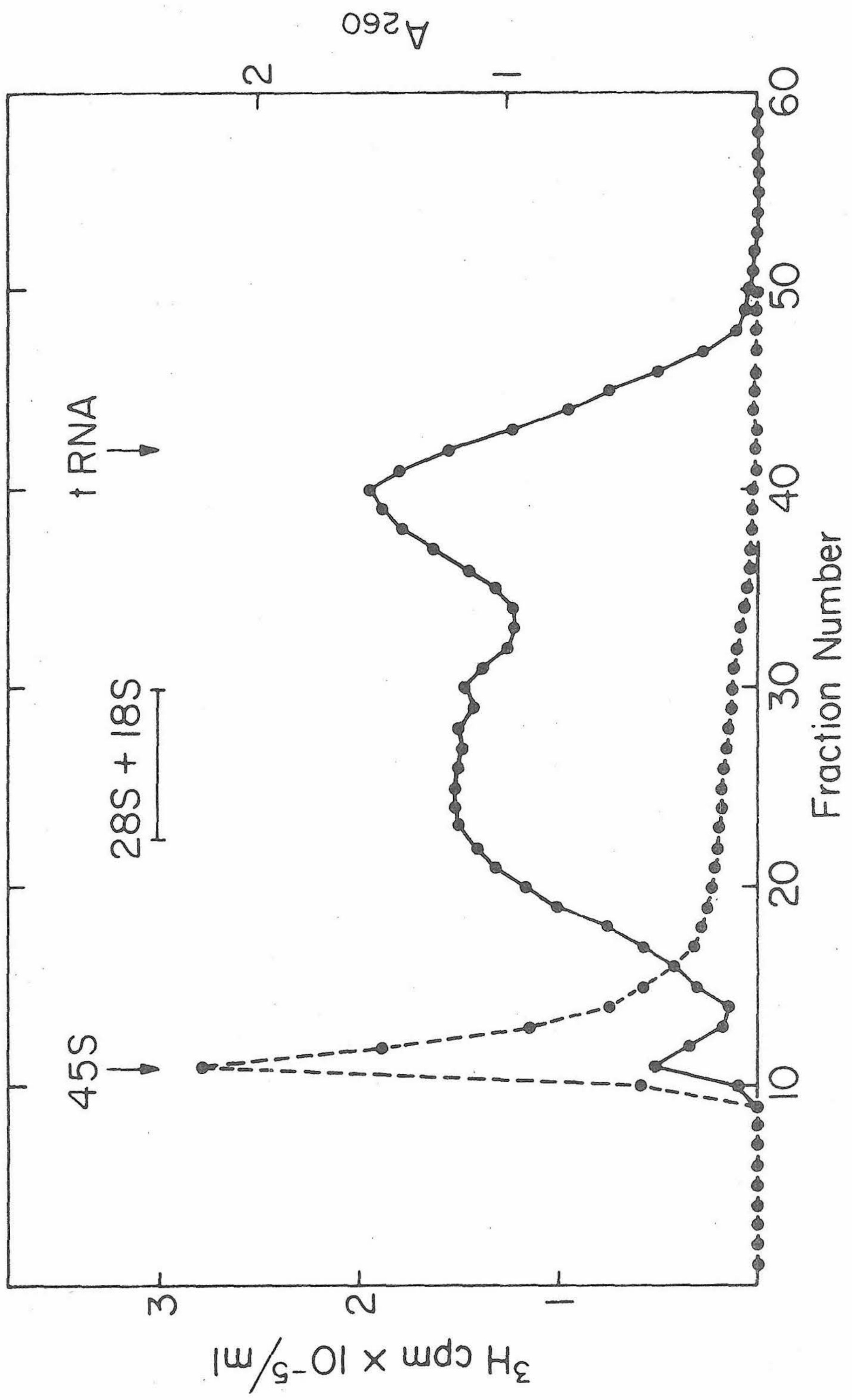
FIGURE 6: Electron micrographs of giant HnRNA prepared by DMSO gradient sedimentation velocity (fractions 11-18 Figure 5a). The RNA was prepared for visualization under the electron microscope essentially by the method of Robberson et al. (1971). The scale shown is 1 μ (about 1.29 x 10^6 daltons). (a)(b) and (d) x 29,000. (c) x 16,000.

FIGURE 7: Frequency and mass distribution of RNA as visualized under the electron microscope. (a) Frequency distribution of 45S rRNA and giant HnRNA prepared by sedimentation through DMSO (giant HnRNA corresponds to fractions 11-18 Figure 5a). (b) Mass distribution of 45S rRNA and giant HnRNA. A total of 98 molecules of 45S rRNA and 252 molecules of giant HnRNA were scored from 5 grids. 15 molecules of 45S rRNA and 42 molecules of giant HnRNA could not be measured due to ambiguity in contour length resulting from incomplete denaturation. Cross hatched area = 45S rRNA prepared from rat nucleoli and open area = giant HnRNA.

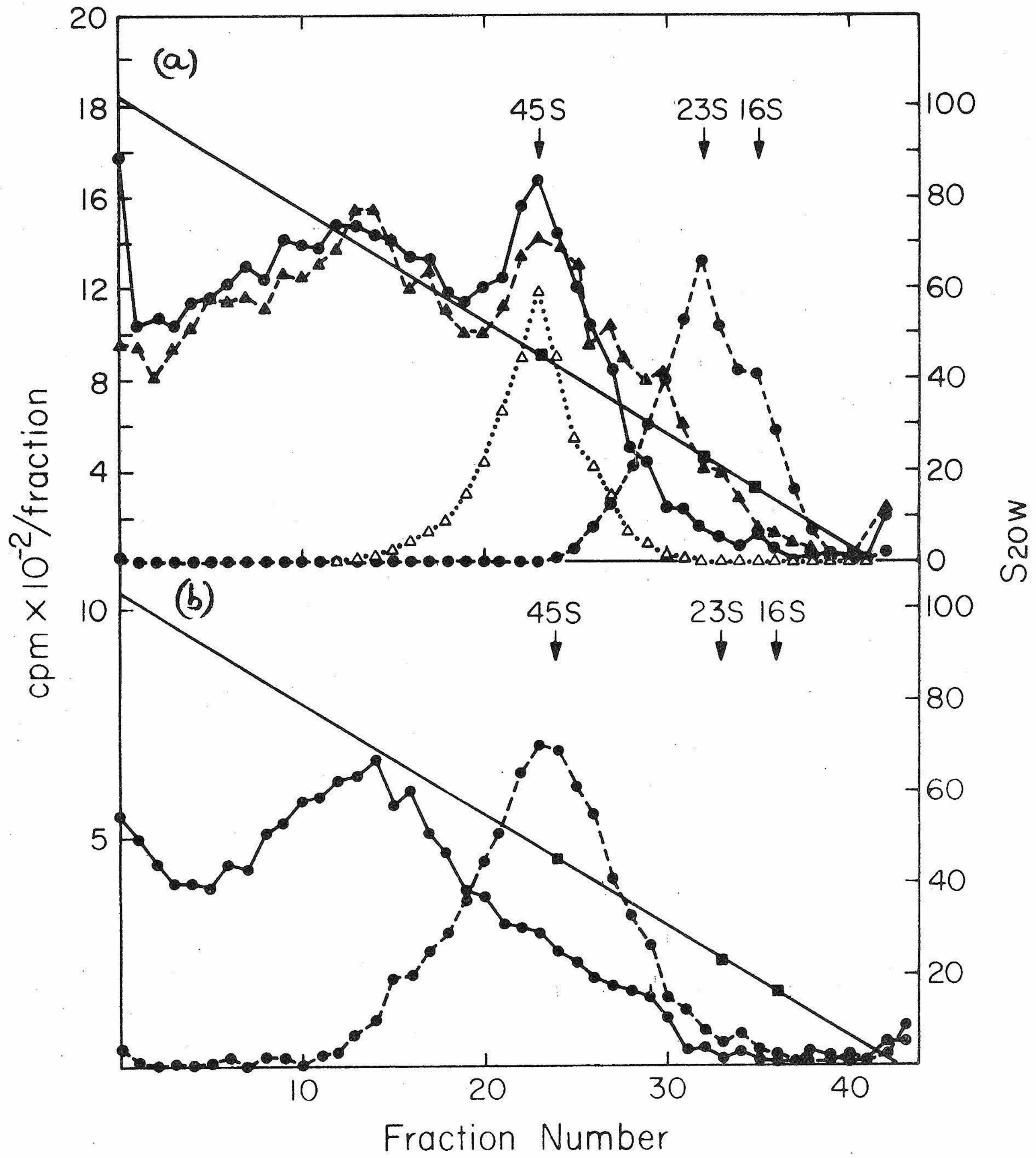
FIGURE 8a: Optical melting profile of RNA in 0.12 M phosphate buffer pH 6.8 recorded in the Gilford automatic recording spectrophotometer equipped with a thermostatically controlled water bath. The temperature was raised automatically at the rate of 0.5°/min. ●——●, sucrose purified giant HnRNA, fractions 0-18 from a gradient similar to that shown in Figure 2b; ■——■, wheat germ 25S + 17S rRNA.

FIGURE 8b: A Van't Hoff plot of the data from Figure 8 a. $A_o = A_{260}$ at 20°. $A_\infty = A_{260}$ at 98°. ●, sucrose purified giant HnRNA, □ , wheat germ 25S + 17S rRNA.

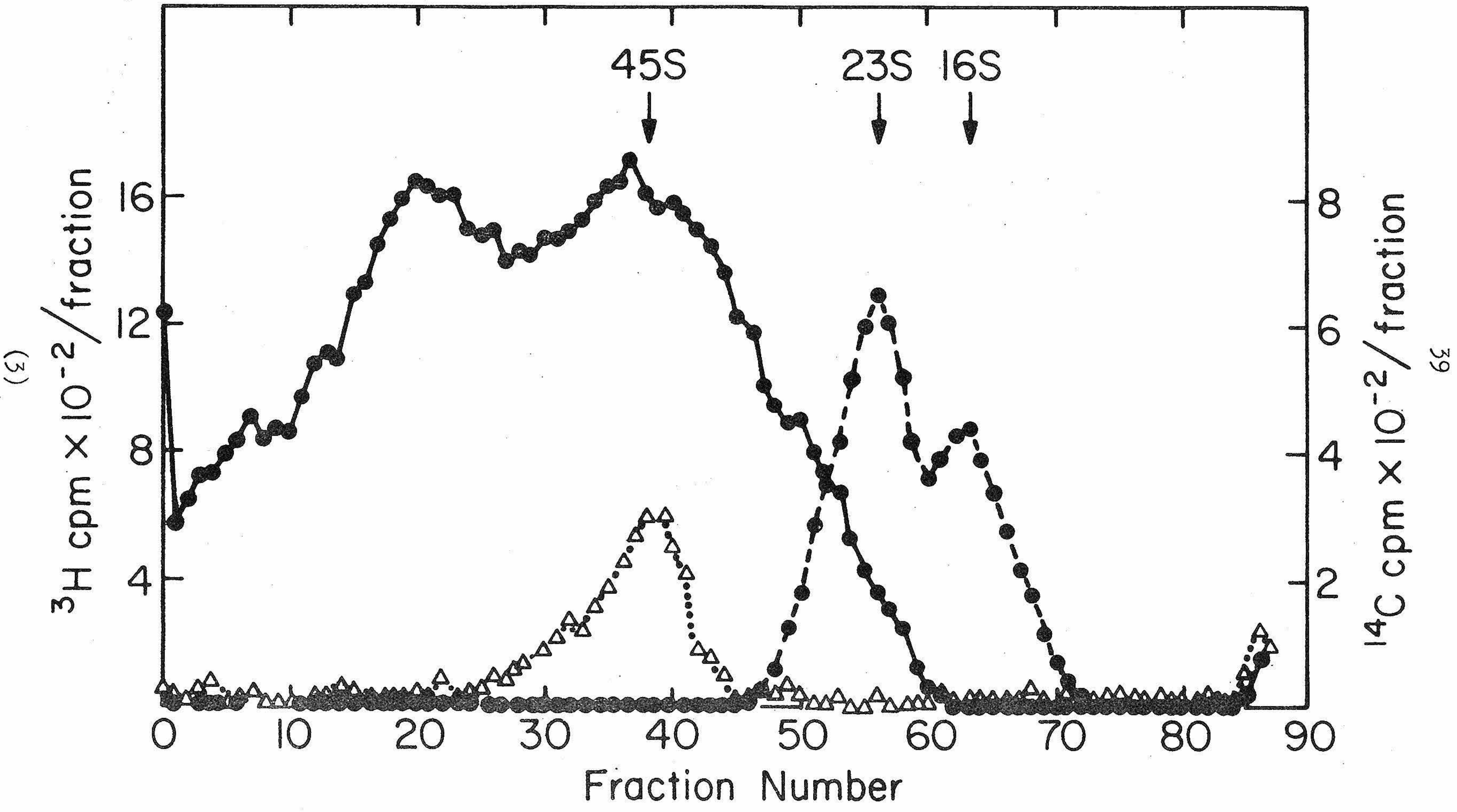
FIGURE 8c: A first order plot for the reaction of sucrose purified giant HnRNA (see Figure 8a) O, and a mixture of ribonucleotides (G = 22%, C = 24%, A = 24%, U = 30%), ● , with 1% formaldehyde (Mallinckrodt reagent grade) in 0.12 phosphate buffer (pH 6.8) at 25°. The reaction was monitored at 275 mμ. $A_o = A_{275}$ at the beginning of reaction. $A_t = A_{275}$ at the appropriate time and $A_\infty = A_{275}$ after equilibration.



(1.)



(2)



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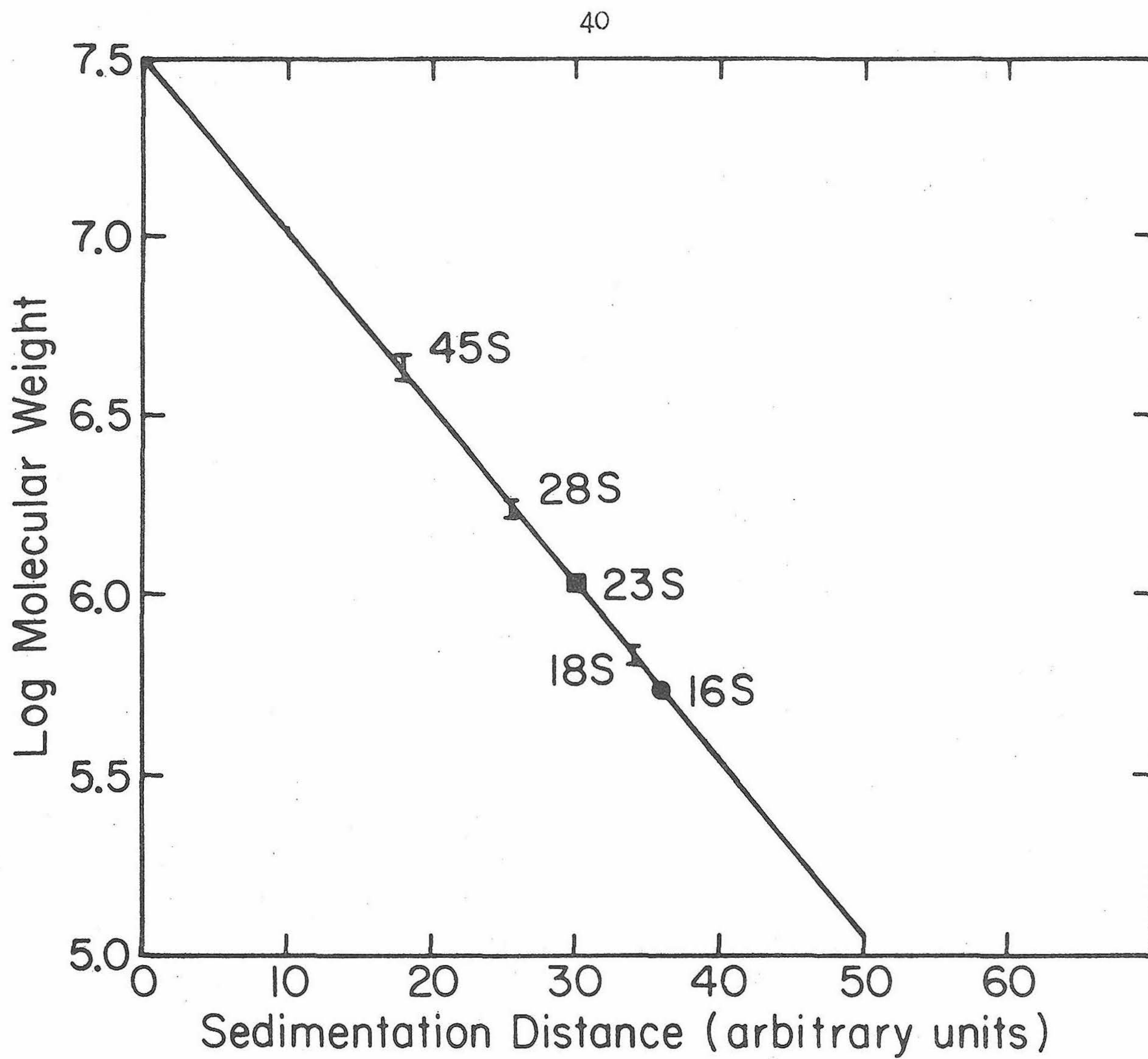

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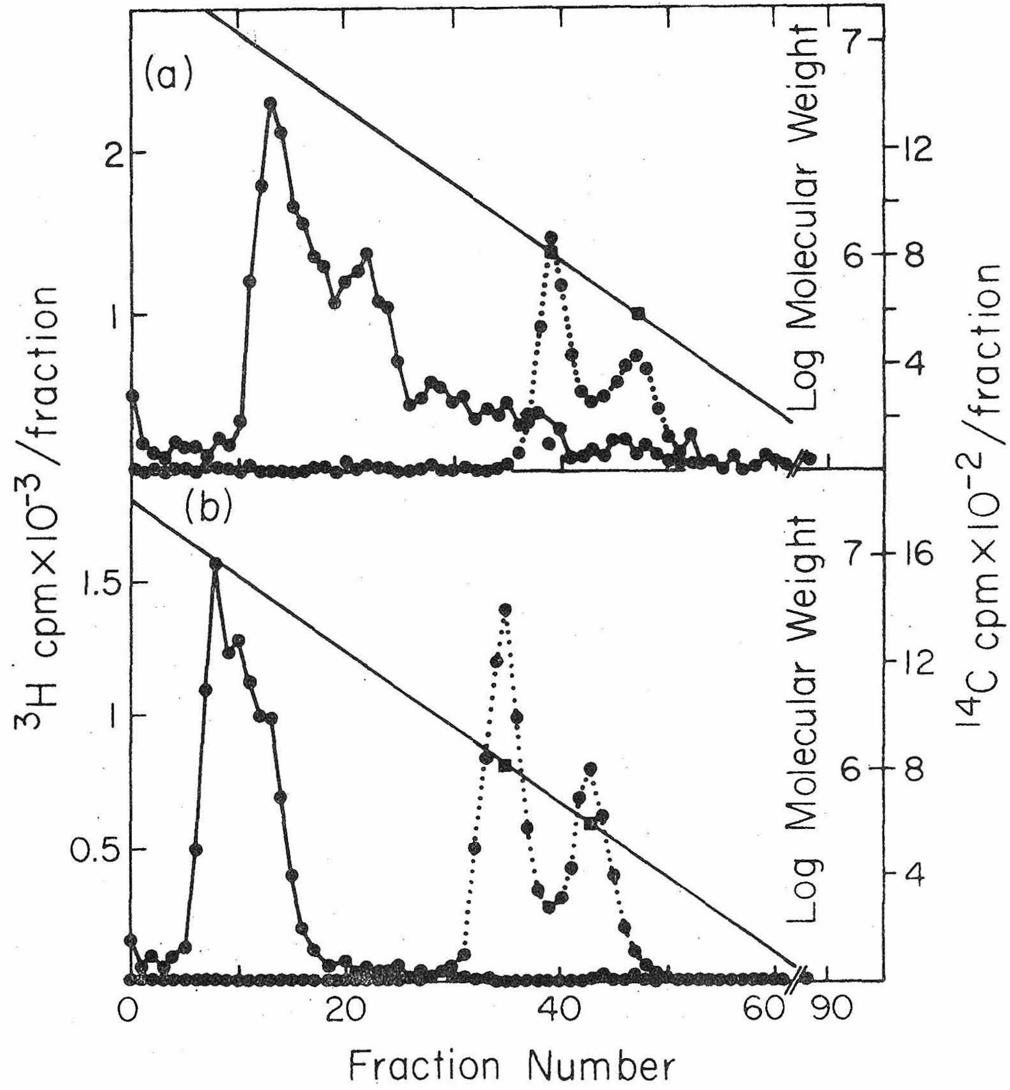

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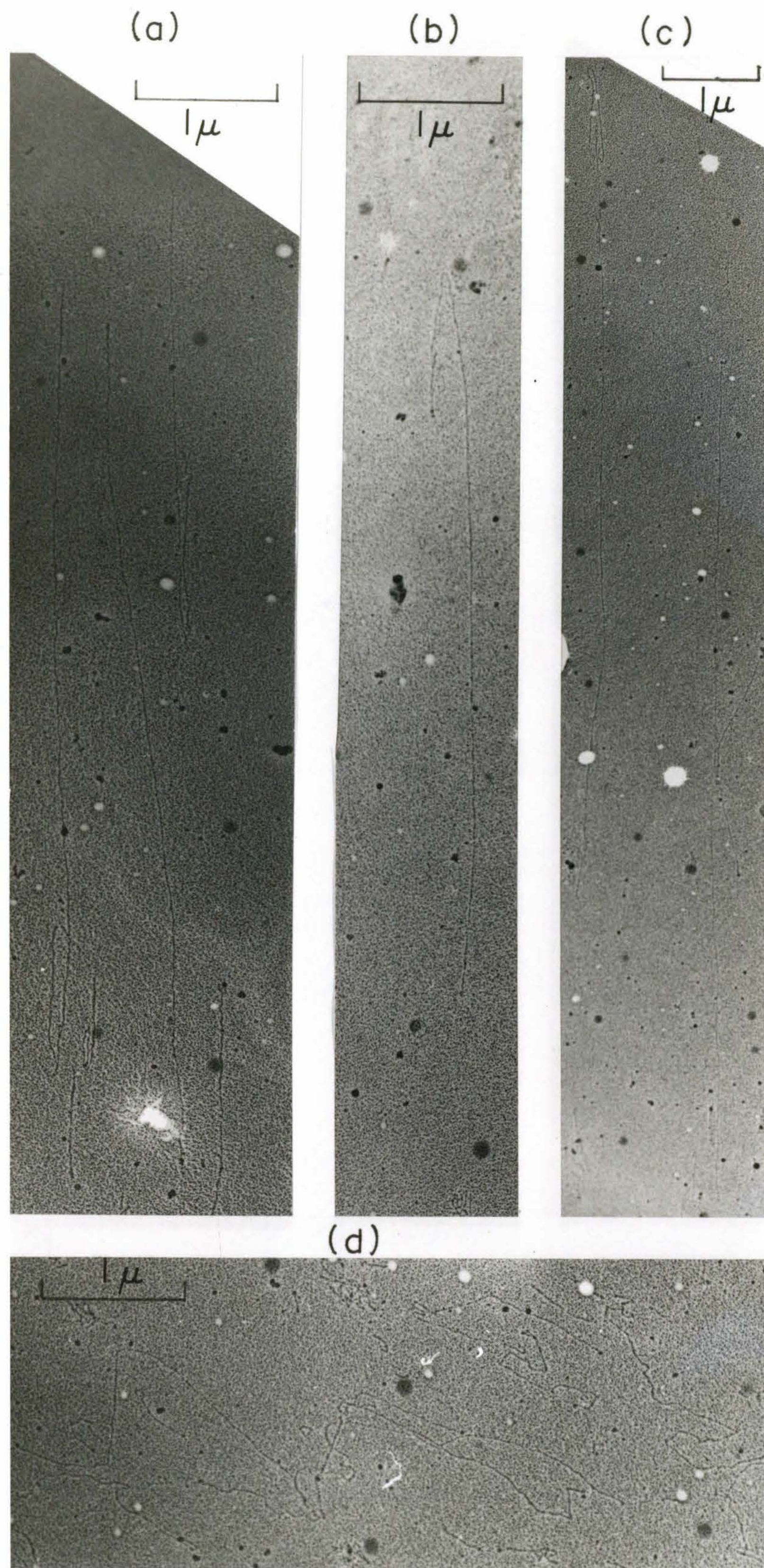
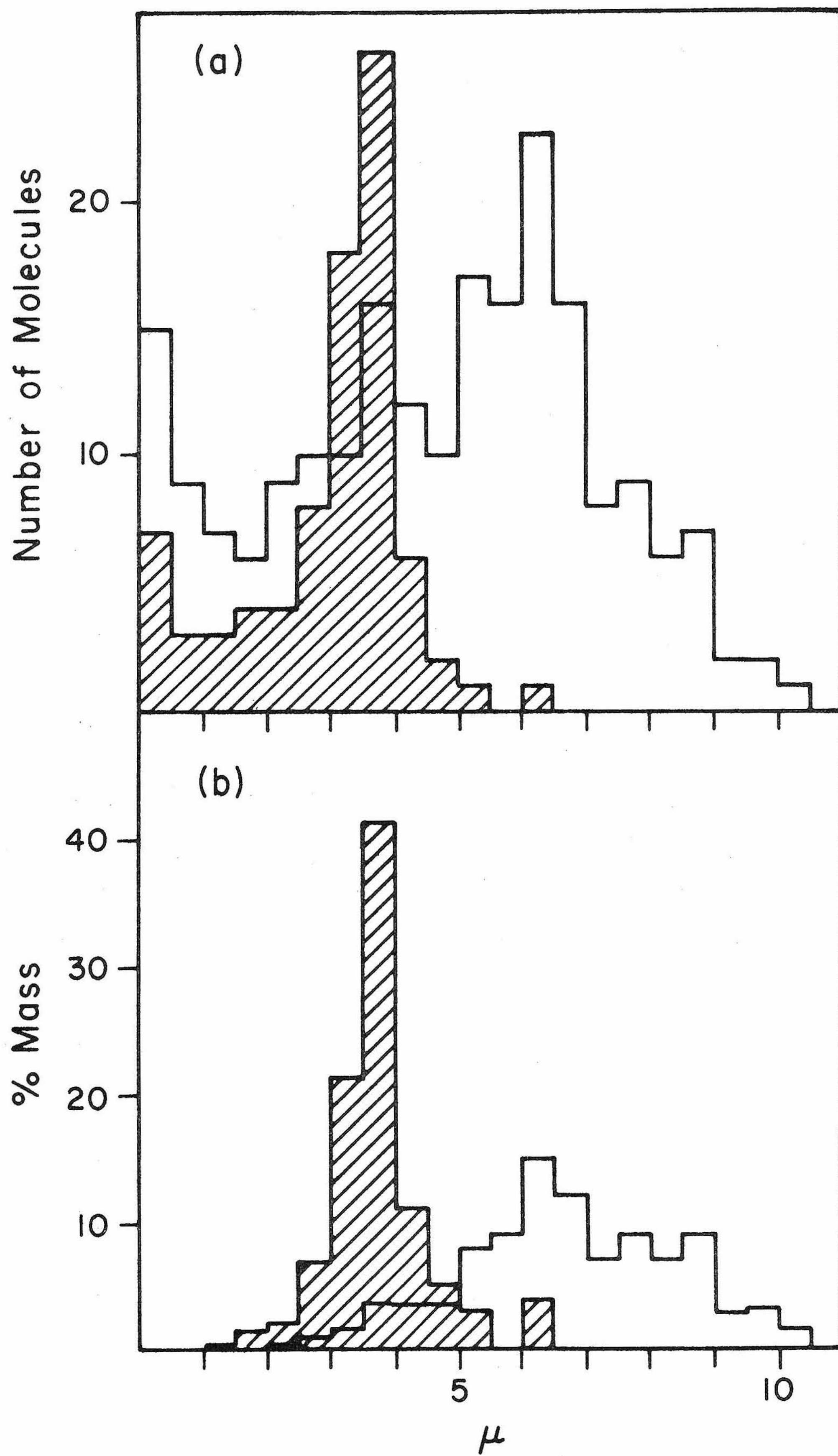
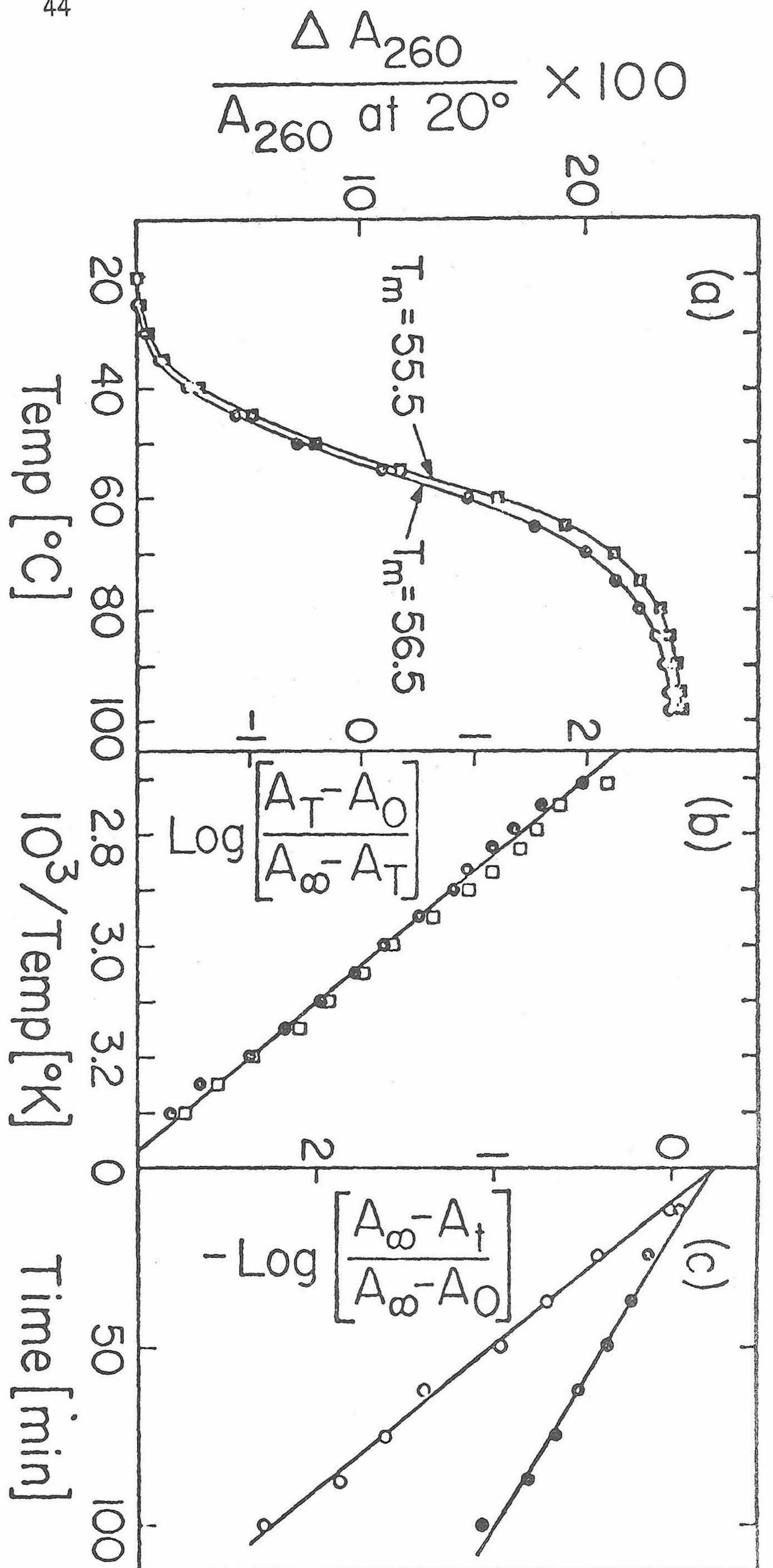

(a)
(b)
(c)
(d)
1μ
1μ
1μ
1μ



(7)



$\frac{\Delta A_{260}}{A_{260} \text{ at } 20°} \times 100$
10
20
(a)
T_m=55.5
T_m=56.5
20
40
60
80
100
Temp [°C]
(b)
Log $\left[\frac{A_T - A_0}{A_\infty - A_T}\right]$
-1
0
1
2
2.8
3.0
3.2
10^3/Temp[°K]
(c)
-Log $\left[\frac{A_\infty - A_t}{A_\infty - A_0}\right]$
0
1
2
0
50
100
Time [min]

CHAPTER 3

The Preparation and Properties of Giant Nuclear RNA

II. Properties of Hybridizable Sequences

ABSTRACT: Rat nuclear DNA is characterized by its reassociation profile ($(Na^+) = 0.18^o$ at 62°, $T_m - 23^o$) as judged by chromatography on hydroxyapatite. Single copy DNA (Cot 1/2 observed = 1.5×10^3) comprised 65% of the genome and 19% of the genome consists of sequences repeated an average 1,800 times (middle repetitive DNA, Cot 1/2 observed = 1.0). 9% of the genome (highly repetitive DNA) reassociates faster than is measured in these experiments (Cot 1/2 observed $< 2 \times 10^{-2}$).

Middle repetitive and single-copy DNA are isolated and characterized with respect to their reassociation kinetics and melting profiles. They reassociate with kinetics similar to the kinetics describing these components when they are present in total genomic DNA. The reassociated single-copy DNA has a high thermal stability indicative of good fidelity in base pairing; the reassociated middle repetitive DNA has a lower thermal stability which is probably attributable, in part, to base-pair mismatch.

Rat nuclear RNA (HnRNA, 5-10 x 10^6 daltons) is hybridized to isolated single copy or middle repetitive DNA ($[Na^+] = 0.18$ at 62°). HnRNA hybridizes to about 4.5% of the single-copy and 9.4% of the middle repetitive DNA. The T_ms of single-copy and middle repetitive hybrids are 1-2° lower than those of the reassociated single-copy and middle repetitive DNA respectively. The DNA isolated from the single-copy or middle repetitive hybrids reassociates with kinetics similar to the input single-copy or middle repetitive DNA respectively. HnRNA is hybridized to total genomic

DNA present in excess. 37% of the HnRNA hybridizes with kinetics (Cot 1/2 = 2.0 x 10^3) similar to single-copy DNA and 12% hybridizes with kinetics (Cot 1/2 = 5.6), a little more slowly than the major reassociating component of middle repetitive DNA.

Introduction.

Nuclei of mammalian cells contain a class of heterogeneous RNA (HnRNA) that is rapidly labeled, sediments heterogeneously in sucrose gradients, and has a DNA-like base composition (Scherrer _et al._, 1963; Attardi _et al._, 1966; Soeiro _et al._, 1966; Schutz _et al._, 1968; Soeiro and Darnell, 1970). Although it appears that the majority of HnRNA is restricted to the nucleus (Perry, 1962; Georgiev _et al._, 1963; McConkey and Hopkins, 1964; Attardi _et al._, 1966; Penman, 1966; Soeiro _et al._, 1966; Yoshikawa-Fukada, 1966; Shearer and McCarthy, 1971) there are several lines of indirect evidence suggesting that a portion of HnRNA is precursor to polyribosomal mRNA (Shearer and McCarthy, 1967; Wagner and Roizman, 1969; Lindberg and Darnell, 1970; Penman _et al._, 1970; Soeiro and Darnell, 1970; Darnell _et al._, 1971; Parsons _et al._, 1971; Philipson _et al._, 1971; McGuire _et al._, 1972). The function of the remainder of the HnRNA is unknown although it could be involved (1) in processing and packaging of particular RNA sequences such as mRNA sequences, (2) in gene regulation, (3) in the production of extra-genomic DNA genes involving reverse transcriptase, or (4) could represent transcripts of particular DNA sequences such as RNA polymerase binding sites or structural genes which are not utilized further and are rapidly degraded.

It is our belief that insight into the function of HnRNA requires an understanding of the composition, and organization, or specific sequences within HnRNA. In a previous paper we showed that under stringent

denaturing conditions a large portion, on a weight basis, of pulse-labeled rat HnRNA consists of molecules in the range 5-10 x 10^6 daltons (Holmes and Bonner, 1973a). This paper analyzes the composition of this HnRNA with respect to various classes of kinetic sequences. The following paper (Holmes and Bonner, 1973b) shows that a small RNA (about 1.6 x 10^4 daltons) associated with isolated chromosomes is enriched in repetitive transcripts some of which are also found in HnRNA.

Materials and Methods

Isolation of DNA and RNA. DNA was isolated from the chromatin of rat Novikoff ascites cells by the method of Dahmus and McConnell (1969). rRNA and giant HnRNA was isolated from rat Novikoff ascites cells as described previously (Holmes and Bonner, 1973a).

Sonication and Labeling of RNA. Giant HnRNA or rRNA was dissolved in 1 mM EDTA pH 8 at a concentration of about 0.25 μg/ml and sonicated, for a total of 2 min in 10-sec pulses with 10-sec intervals for cooling in ice, using a Branson micro-tip sonicator. The sonicated RNA was precipitated at -18° for 4 hr in 2% sodium acetate pH 5 (v/v) and 2 vols of 95% ethanol, then dissolved in 0.01 M sodium acetate pH 6, 0.1 M NaCl, 1 mM EDTA, 0.2% SDS. 1-ml samples of the RNA at a concentration of *ca.* 0.5 mg/ml were loaded onto 5-20% sucrose gradients (Mann ultrapure sucrose) made in the same buffer and centrifuged for 16 hr at 25,000 rev/min in the Spinco SW-25.2 rotor at 18°. Following centrifugation the tubes were dripped and fractions corresponding to RNA of 4-8 S were pooled and precipitated as described above. (*E. coli* 23 S and 16 S rRNA were used as markers.) The RNA was dissolved in 0.1 M

potassium phosphate buffer pH 7.4 at a concentration of *ca.* 1 mg/ml and labeled *in vitro* with ^{3}H-dimethyl sulfate (New England Nuclear, 2300 Ci/M), essentially by the method of Smith *et al.* (1968). 200 µl of the RNA were added to 5 mC of ^{3}H-dimethyl sulfate in the same vial as the isotope was purchased (care was taken to ensure that all ^{3}H-dimethyl sulfate on the walls and top of the vial were dissolved in buffer), and incubated overnight at 4°. Unreacted ^{3}H-dimethyl sulfate was removed by Sephadex G-50 chromatography followed by precipitation of the excluded RNA with ethanol as described above. The resulting specific activities were 110,000 cpm/µg (4% of the total bases methylated) for rRNA and 76,000 cpm/µg (3% of the total bases methylated) for HnRNA (% methylation corrected for quenching).

Purification of DNA Components and Labeling of DNA. DNA (about 1 mg/ml) was dissolved in 0.06 M phosphate buffer pH 6.8 and sheared to about 350 nucleotides (single-strand length judged by electron microscopy) by two passages through a Rib-Sorvall cell fractionator at 50,000 psi. Purified DNA components were prepared by incubation of the DNA in phosphate buffer pH 6.8 (for conditions see legend to Fig. 1) to an appropriate equivalent Cot followed by separation of double- from single-stranded DNA by passage through hydroxyapatite chromatography as described by Britten and Kohne (1967). Single-copy DNA was prepared by 2 cycles of reassociation followed by isolation on hydroxyapatite of the DNA which remained unreacted at a Cot of 2×10^3. Middle repetitive DNA was isolated by separation of the DNA which reassociated between Cot 2×10^{-2} and Cot 10^2. The highly repetitive DNA which reacted before Cot 2×10^{-2} (*ca.* 9% of the total) was discarded.

Purified single-copy and middle repetitive DNA were labeled _in vitro_ with ^{125}I by the method of Commerford _et al_. (1971). The resulting specific activities were 178,000 cpm/μg for middle repetitive DNA and 166,000 cpm/μg for single-copy DNA. These specific activities correspond to about 0.1% iodination of the total bases.

Hydroxyapatite Chromatography. Chromatography of DNA on hydroxyapatite was carried out by the method of Britten and Kohne (1967). One ml of packed hydroxyapatite (Biorad) was used per 100 μg of DNA applied and the DNA eluted with 6 column volumes of the appropriate buffer. The eluted DNA was precipitated in cold 10% TCA (trichloroacetic acid) at 4° for 10 min in the presence of yeast soluble RNA (CalBiochem) added to a final concentration of 50 μg/ml nucleic acids. The filters were washed with cold 10% TCA followed by 60% ethanol, then dried and dissolved in 1 ml of ethyl acetate, and counted in a toluene scintillation cocktail.

RNA Excess Hybridization. RNA was prepared via sedimentation in aqueous sucrose gradients and RNA $\geqslant$ 60 S selected as described by Holmes and Bonner (1973a). Unlabeled sonicated RNA was added to single-copy ^{125}I-DNA and hybridization carried out in either 0.12 M phosphate buffer pH 6.8-1 mM EDTA at 62° or in 0.48 M phosphate buffer pH 6.8-1 mM EDTA at 66°. The concentration of RNA and DNA and the length of incubation are recorded in the legend to Figure 4. At the end of incubation the reaction mixes were diluted to 0.12 M phosphate buffer pH 6.8 and passed through a column containing hydroxyapatite equilibrated with 0.12 M phosphate buffer pH 6.8 at 62°. The column was washed with 0.12 M

phosphate buffer pH 6.8 to remove single-stranded DNA followed by 0.48 M phosphate pH 6.8 to remove DNA-RNA hybrids. The radioactivity associated with DNA was measured as described above.

Some formation of DNA-DNA complexes occurs during the course of the reaction. This was estimated and subtracted from the estimates of RNA-DNA hybrids as described by Hough and Davidson (1973). DNA was isolated from the RNA-DNA hybrids and its reassociation kinetics estimated as described by Hough and Davidson (1973).

DNA Excess Hybridization. Sonicated *in vitro* labeled ^{3}H-HnRNA (prepared through DMSO, Holmes and Bonner, 1973a), or ^{3}H-rRNA were reacted with total nuclear DNA (sheared to about 350 bases single-strand length). The DNA to rRNA ratio was about 319,000:1 and the DNA to HnRNA ratio was about 170,000:1. The conditions of the reaction are described in the legend to Figure 6. At the end of incubation the solution was divided into two aliquots. One aliquot was adjusted to 0.24 M phosphate buffer pH 6.8 and subjected to treatment with 20 µg/ml ribonuclease A and 20 units/ml ribonuclease T1 at 37° for 15 min. The solution was precipitated with TCA and collected on filters as described by Melli *et al*. (1971). The filters were washed and the radioactivity estimated as described above. The other aliquot was used to measure DNA reassociation by chromatography on hydroxyapatite as described above, using the A_{260} of the eluant to estimate DNA content.

Melting Profiles. Purified middle repetitive DNA was reassociated to a Cot of 10^{2} and single-copy DNA to a Cot of 2×10^{4} as described above and applied in 0.12 M phosphate buffer pH 6.8 to hydroxyapatite, equilibrated at 55°. The column temperature was raised at 5° increments and

allowed to equilibrate for 5 min at each step. Single-stranded DNA was eluted with 0.12 M phosphate buffer pH 6.8, precipitated with TCA onto filters and counted as described above. The melting profile of RNA-DNA hybrids was estimated in the same manner. Hybrids for melting were taken from the final hybridization points shown in Figures 3a and 3b.

Results

Sequence Composition of Rat Nuclear DNA. The point of departure of these studies is an analysis of the sequence composition of rat nuclear DNA. This analysis is a necessary prerequisite to the identification and isolation of kinetic components of nuclear DNA which will be described in the next section. Figure 1 shows the reassociation profile of sheared rat nuclear DNA determined by the method of Britten and Kohne (1967). Second order reaction curves have been fitted to the major reassociating components. Table I lists the parameters which describe these reaction curves. These parameters depend, in part, on the conditions used to determine duplex stability (Britten and Kohne, 1968; McCarthy and Duerksen, 1970). At the criterion of the reassociation chosen (0.18 $[Na]^+$, 62°, T_m-23°) about 65% of the input DNA reassociates with kinetics indicating that each sequence is present about once per haploid genome and 19% reassociates with kinetics indicating that each sequence of the major component of this class is repeated about 1800 times per haploid genome. We define this DNA (reassociating between a Cot of 2×10^{-1} and 10^2) as "middle repetitive." About 9% reassociates

faster than can be measured in this experiment. This component may represent DNA with internal strand homology or may correspond to the fast-reassociating satellite found in other organisms (Waring and Britten, 1966; Kram *et al.*, 1972).

Isolation of Purified Single Copy and Middle Repetitive DNA. The strategy for the isolation of purified single-copy and middle repetitive DNA is based on the reassociation of total DNA to a particular Cot and the separation of DNA which remains single-stranded from that which has reassociated at that Cot. The success of this strategy depends in part on the size of the DNA fragments which are allowed to reassociate and in part on the magnitude of the difference in the reaction rate between the particular components. In these experiments the DNA was sheared to an average of 350 bases (single-strand length) which is approximately the modal average of the middle repetitive sequences and below that of the single copy sequences (about 800 bases) of rat DNA (Wu *et al.*, 1973). Thus, if we assume random shearing of the DNA, each fragment of DNA will contain predominantly either repetitive or single-copy sequences; few, if any, of the fragments of DNA will contain a repetitive sequence with considerable covalently attached single copy DNA or vice versa. The difference between the second order reaction rate describing the single copy ($k = 7.2 \times 10^{-4}$) component and that describing the major component of the middle repetitive DNA ($k = 9.8 \times 10^{-1}$) is about three orders of magnitude.

The efficiency with which these components can be isolated is shown in Figure 2. In this experiment the isolated single-copy and

middle repetitive components have been labeled *in vitro* with ^{125}I and reassociated with excess unlabeled total DNA. The reaction rates describing the major component of the middle repetitive DNA ($k = 7.2 \times 10^{-1}$) and the single copy DNA ($k = 6.1 \times 10^{-4}$) are in good agreement with the reaction rates of these components estimated from the reassociation profile of total DNA (Table I). The second order reaction curves that have been fitted to the reassociation profile of purified middle repetitive and single copy DNA (see Figure 2) describe the data sufficiently well to indicate that no gross selection for kinetic components other than the major ones has taken place during the isolation of single copy and middle repetitive DNA. This is particularly germane with respect to the middle repetitive DNA which probably contains minor fractions of DNA which reassociate at rates different from that describing the major fraction. Also shown in Figure 2 is the reassociation of DNA isolated from DNA-RNA hybrids. We will describe these data in the next section.

The melting profiles of the reassociated purified single copy and middle repetitive DNA are shown in Figure 3. The single copy DNA melts with a T_m about 2° below that of native DNA indicating excellent fidelity of base pairing. Compared to native DNA the melting profile of single copy DNA has a distinct "foot" of material melting between 60° and 75°. This foot can probably be accounted for by the brevity of some of the duplexes resulting from the shortness of some of the single copy sequences due to random shearing. These observations are in accord with those of other workers (Britten and Kohne, 1967; Brown and Church, 1971; Hahn and Laird, 1971; Firtel and Bonner, 1972). The middle repetitive DNA

has a broader, less cooperative, melting profile, with a T_m about 10° lower than native DNA. Part of this lowering of T_m might be attributable to the extreme shortness of the repetitive duplex regions (Wu *et al.*, 1973), as suggested by the findings of Hayes *et al.* (1970). A portion of the lowering of T_m is probably due to base pair mismatch occurring during reassociation as suggested by the reduced hyperchromicity of the repetitive components compared to native DNA of other systems and its sensitivity to manipulation of reaction parameters (Britten and Kohne, 1968; McCarthy and Duerksen, 1970). We do not know to what extent, if any, the melting profile of the middle repetitive DNA is a result of its base composition.

Since the reassociation rates of the purified components are close to those predicted from the parameters of Table II, and since the single copy duplexes have a T_m close to that of native DNA, it is unlikely that iodination of these components has had a marked effect on their reassociation rate or the stability of the resulting duplexes.

Hybridization of HnRNA to Middle Repetitive or Single Copy DNA. Purified single-copy or middle repetitive ^{125}I-DNA was allowed to react with excess HnRNA under conditions allowing the formation of DNA-RNA hybrids. At certain times during the reactions hybrids were assayed by passage of the reaction mixture over hydroxyapatite. Since this method scores the ^{125}I present in DNA as either DNA-DNA or DNA-RNA duplexes there is no absolute requirement for prior ribonuclease treatment of the reaction mixture. The results of this experiment are shown in Figure 4. A certain amount of DNA-DNA reassociation takes place during the course of the reaction. This can be assayed as described in Methods. Briefly

the strategy calls for extensive ribonuclease treatment of an aliquot of the reaction mixture such that the only surviving duplexes are DNA-DNA.

The data displayed in Figure 4a,b indicate that HnRNA is capable of forming duplexes with ^{125}I-DNA to an extent such that at least 9.5% of the middle repetitive DNA or 4.5% of the single-copy DNA is retained on hydroxyapatite under conditions where duplex but not single-stranded DNA is retained. These values probably yield a reasonable estimate of hybrid formation. Although some of the DNA fragments in duplex form will be longer than the "true" length of the DNA site complementary to the RNA, random shearing of the DNA will compensate for this effect by the production of DNA fragments which contain complementary RNA sites too short to form hybrids at the criterion of hybridization used.

The rate of hybridization of HnRNA to middle repetitive and single copy DNA roughly estimates the proportion of repetitive and single copy transcripts in the RNA. From fig 4 half-reactions occur at 0.14 mg/ml hr^{-1} (6.3×10^3 mol - L - sec^{-1}) for middle repetitive and single copy hybrids respectively, yielding pseudo first order rate constants of 0.46 and 1.0×10^{-4}. From the rate of formation of middle repetitive and single copy DNA duplexes it is estimated that the rate constants for the formation of hybrids with these DNA components should be 22 and 1.0×10^{-2} respectively. Therefore about 2% of the RNA consists of middle repetitive transcripts and 1% consists of single copy transcripts. However these estimates will be affected by parameters that have not at present been evaluated such as the viscosity of the reaction mixture due to high concentrations of RNA and variations in the concentrations of different RNA sequences.

Figure 5 shows the melting profiles of single-copy and middle

repetitive DNA-RNA hybrids as determined by thermal elution from hydroxyapatite. These profiles are similar to, respectively, the single-copy DNA and the middle repetitive DNA melting profiles shown in Figure 3. The single copy DNA-RNA hybrids melt with a T_m about 3° below the T_m for native DNA indicating excellent fidelity in base pairing, in agreement with the results of others on the melting of single-copy DNA hybrids (Davidson and Hough, 1969; Hahn and Laird, 1971; Brown and Church, 1971; Gelderman et al, 1971; Grouse et al, 1972; McConnaughy and McCarthy, 1971; Firtel, 1972). The middle repetitive DNA-RNA hybrids melt with a T_m about 12° below that of native DNA. It is not known to what extent the reduced T_m is due to base pair mismatch, to shortness of hybrid duplexes (Hayes et al, 1970) or to base composition effects. Since 97% of the middle repetitive ^{125}I-DNA and 92% of the single copy ^{125}I-DNA-DNA duplexes are present in hybrid structures, the contribution of ^{125}I-DNA-DNA duplexes to the melting profile is negligible.

To test if the observed hybridization takes place with a subfraction of DNA sequences that have relative concentrations different from the major reassociation components of the purified DNA classes, ^{125}I-DNA was isolated from either single-copy or middle repetitive duplex structures and reassociated in the presence of excess unlabeled total DNA as described in a previous section. The results are shown in Figure 2. The DNA from the hybrids reassociates with approximately the same kinetics as the input DNA. These data are consistent with the view that the middle repetitive DNA complementary to HnRNA contains sequences present at approximately the same degree of repetition as the major reassociating component of the input middle repetitive DNA and that the majority of the hybridization of HnRNA to

purified single-copy DNA is to bona fide single-copy sequences or to sequences of low reiteration. In the limit each middle repetitive sequence will have one representative in the single-copy DNA but the hybridization to these middle repetitive representations will not contribute significantly to the overall observed hybridization. As a rough estimate, only 0.02% of the hybridization to the single-copy DNA can be attributed to hybridization to representatives of the middle of the middle repetitive DNA ($\frac{2/2000}{4}$ x 10^2

$$\frac{\text{\% total DNA hybridized by repetitive transcripts/no. copies per genome}}{\text{\% total DNA hybridized by single copy transcripts}})$$

Hybridization of Giant HnRNA to Nuclear DNA Present in Vast Excess. In the presence of a vast excess of sheared DNA the rate of hybridization of RNA is predominantly governed by the concentration of complementary DNA sequences (Gelderman et al., 1971; Melli and Bishop, 1971). In the experiment shown in Figure 6 sonicated giant HnRNA and rRNA were reacted with an excess of sheared total nuclear DNA. The parameters which describe the hybridization reactions of these RNA species are listed in Table II. The estimates of the repetitive frequencies of the kinetic components of the giant HnRNA are only approximations. Several unquantitated parameters such as possible variations in base composition, secondary structure of the RNA, sensitivity of the hybrids to ribonuclease, base pair mismatch, and differences in the ratio of complementary DNA sequences to RNA across the Cot curve could all contribute to the determination of the relative proportions and reaction rates of the observed components. In qualitative terms the major part of the HnRNA hybridization occurs in the late part of the reaction with kinetics (Cot 1/2 = 2.08 x 10^3) similar to those describing the reassociation of the single-copy DNA (Cot 1/2 = 1.52 x 10^3). A portion

of the observed hybridization (about 22% of the reacting RNA or 12% of the input RNA) occurs in the middle section of the reaction. Assuming that this part of the hybridization can be described by a single second order curve, the Cot 1/2 (5.65) of this component is a little greater than that describing the major reassociating component of the middle repetitive DNA (Cot 1/2 = 1.06). The observed hybridization to the middle repetitive DNA is probably not due to contamination of the HnRNA with rRNA because the reaction rates of these two classes of RNA under the same experimental conditions differs by a factor of about 6.

Discussion

The experiments described in this paper strongly argue for the occurrence of both repetitive and single-copy transcripts in giant HnRNA under specific conditions defining duplex stability. Previous reports from other groups have demonstrated the occurrence of both classes of transcripts in whole cell or total nuclear RNA (Gelderman _et al_., 1971; Hahn and Laird, 1971; Davidson and Hough, 1969; Hough and Davidson, 1973) and it seems reasonable to suppose that this would also be the case with giant HnRNA. However, this paper represents the first experimental verification of this supposition, using HnRNA demonstrated to be of very high molecular weight under stringent denaturing conditions.

The informational content of the giant HnRNA is remarkably large. At least 4.3% of the single-copy DNA or 3% of the total DNA is complementary to giant HnRNA. Assuming asymmetric transcription, this represents about 9.6×10^7 base pairs of DNA.

With respect to middle repetitive transcripts the quantitative

information that can be extracted from these experiments is less reliable. This is due to parameters affecting the reaction that cannot at present be evaluated, such as variations in the concentration of sequences and variations in the specificity of hybridization dictated by the conditions used to define duplex stability. With these caveats in mind only rough approximations about the information content of the middle repetitive transcripts can be made. About 10% of the middle repetitive DNA or 2% of the total DNA is complementary to giant HnRNA. Assuming asymmetric transcription this represents about 6.4×10^7 base pairs of DNA.

The function of the individual repetitive and single-copy sequences in giant HnRNA is an important, and as yet unresolved, question. Indirect evidence suggests that some HnRNA is a precursor to polysomal mRNA.

Some polysomal mRNAs encoding specific cell products have been shown to be products of the single-copy DNA or DNA of very low repetition frequency (Suzuki _et al._, 1972; Harrison _et al._, 1972). Thus it is expected, but not proven, that some of the single-copy sequences of giant HnRNA might represent precursor to mRNA. The remainder of the sequences, many of which do not leave the nucleus, are of unknown function, although it is speculated that these sequences could be involved in post-transcriptional processing or packaging of the RNA, or could represent the transcriptional product of sequences involved in the mechanics of transcriptional regulation.

Several reports have discussed the possibility that Dipteran puffs might be responsible for the production of discrete HnRNA molecules and that, by implication, the chromomeres of Dipteran

polytene chromosomes might represent the structural manifestations of a unit of transcription (Berendes, 1968; Grossbach, 1969; Daneholt, 1973; Lambert, 1973). Since the molecular weight of a portion of rat HnRNA (5-10 x 10^6 daltons) corresponds to the expected molecular weight of the giant RNA transcripts of Dipteran puffs (Edström and Daneholt, 1967; Daneholt, 1973) it is conceivable that the mammalian genome is organized into units of transcription homologous to those of Diptera.

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Figure Legends

FIGURE 1: Computer analysis of the renaturation profile of total nuclear rat ascites DNA. Denatured sheared DNA (ca. 350 nucleotides in length) was reassociated in either 0.12 M □ , or 0.48 phosphate buffer ■ , at 62° or 66° respectively. Reassociation was measured by passage of the reacted DNA through hydroxyapatite as described by Britten and Kohne (1967). The computer program was supplied by Dr. R. J. Britten. The root mean square of the line describing the reassociation of the total DNA is 0.025, the reassociation of the DNA goes to 94% completion.

FIGURE 2: Cot plot of trace quantities of single-copy and middle repetitive ^{125}I-DNA in the presence of unlabeled total nuclear DNA. A ratio of about 1300:1 total nuclear DNA to ^{125}I-DNA was used. Reassociation was carried out in 0.12 M or 0.48 M phosphate buffer pH 6.8 at 62° or 66° respectively and the extent of reassociation measured by passage of the reaction mixture through a hydroxyapatite column as described by Britten and Kohne (1967). Purified middle repetitive ^{125}I-DNA ■ ; purified middle repetitive ^{125}I-DNA isolated from DNA:HnRNA hybrids □ ; purified single-copy ^{125}I-DNA, O ; purified single-copy ^{125}I-DNA isolated from DNA:HnRNA hybrids, ●; a second order reaction curve has been fitted to the single-copy ^{125}I-DNA and to the major reassociating fraction of the middle repetitive ^{125}I-DNA. The ^{125}I-DNA was isolated from DNA-HnRNA hybrids as described by Hough and Davidson (1973).

FIGURE 3: Integral melting curve of DNA by thermal elution from hydroxyapatite as described by Britten and Kohne (1966). The DNA was applied in 0.12 M phosphate buffer pH 6.8 to a jacketed hydroxyapatite column equilibrated at 55°. The percent DNA eluted after exhaustive washing at 55° in 0.12 M phosphate buffer is shown by the bar at the left. The column temperature was raised in 5° increments, equilibrated at the temperature for 5 min and then washed with 6 column volumes of 0.12 M phosphate buffer pH 6.8. The eluant was either precipitated and counted as described in Methods in the case of ^{125}I-DNA or read at A_{260} in the case of native DNA. ●——●, native DNA; ■ —— ■, reassociated single-copy ^{125}I-DNA; ▲——▲, reassociated middle repetitive ^{125}I-DNA.

FIGURE 4: Hybridization of unlabeled rat HnRNA (sonicated to about 4-8 S in size) to ^{125}I-labeled purified kinetic components of rat nuclear DNA (sheared to about 350 bases single-strand length). The extent of hybridization was estimated by chromatography of the reaction mix on hydroxyapatite as described in Methods. (a) Hybridization to middle repetitive DNA. The reaction was carried out in 0.12 M phosphate buffer pH 6.8 - 1 mM EDTA at 62°. The specific activity of DNA was about 178,000 cpm/μg and the RNA to DNA ratio was about 4,500:1.

RNA concentration (mg/ml)	Hours incubation	% DNA in DNA-RNA hybrids	%DNA in DNA-DNA duplexes*
0.105	0.2	1.98	2.8
0.105	0.8	3.0	2.1
0.105	1.4	4.3	2.6
0.495	0.6	6.8	2.2
0.495	1.2	8.05	2.1
0.495	2.0	9.4	2.3

FIGURE 4 (continued)

(b) Hybridization to single copy DNA. The reaction was carried out in 0.48 M phosphate buffer pH 6.8 - 1 mM EDTA at 66°. The specific activity of the DNA was about 166,000 cpm/µg and the RNA to DNA ratio was about 1011:1.

RNA concentration (mg/ml)	Hours incubation	% DNA in DNA-RNA hybrids	%DNA in DNA-DNA duplexes*
10	9	1.21	2.8
10	18	2.81	3.4
10	24	3.19	5.65
10	36	3.8	6.98
10	54.5	4.22	8.05
10	96	4.49	8.1

*DNA-DNA reassociation was measured by the method of Hough and Davidson (1973).

FIGURE 5: Integral melting curve of ^{125}I-DNA hybrids assayed as described in the legend to Figure 3. ●——●, single copy ^{125}I-DNA hybrids; ■ —— ■, middle repetitive ^{125}I-DNA hybrids.

FIGURE 6: Computer analysis of the hybridization of sonicated ^{3}H-RNA to nuclear DNA (sheared to about 350 bases single-strand length). The reassociation of DNA has been included for comparison. Squares represent DNA reassociation; circles represent ^{3}H-HnRNA hybridization; triangles represent ^{3}H-rRNA hybridization. The ratio of DNA to ^{3}H-HnRNA was about 170,000:1 and that of DNA to ^{3}H-rRNA was about 319,000:1 (ratio of rDNA to rRNA about 90:1). Hybridization was carried out in either 0.12 M

FIGURE 6 (continued)

phosphate buffer pH 6.8 (open symbols) or 0.48 M phosphate buffer pH 6.8 (closed symbols) at 60° or 66° respectively. The reassociation of DNA was monitored by passage of an aliquot of the reaction mixture through hydroxyapatite as described by Britten and Kohne (1967). After mild ribonuclease treatment as described in Methods the RNA:DNA hybrids were monitored by the TCA precipitation method of Melli _et al._ (1971) as described in Methods. The rate parameters describing the hybridization of rRNA to DNA in excess as judged by TCA precipitability are comparable to those determined by hydroxyapatite binding (Straus and Bonner, 1972). In control solutions in which RNA was incubated without DNA, about 8-16% of the RNA remained ribonuclease-resistant at the end of ribonuclease treatment. This has been subtracted as background from each of the points presented in this figure. At a Cot of 10^4, 5-15% of the input RNA was TCA-insoluble. The computer program was supplied by Dr. R. J. Britten. The root mean square of the line describing the hybridization reaction is 0.021.

TABLE I: Composition of Rat Nuclear DNA with Respect to Components of Various Degrees of Repetition

Class of DNA	% total DNA	Cot 1/2 (observed)	Average Cot 1/2 pure (estimated)	Average kinetic complexity (daltons) relative to E.coli (a)	daltons per genome (b)	repetition
Non-repetitive	66	1.6×10^{3}	1.1×10^{3}	0.96×10^{12}	1.17×10^{12}	1.2
Middle repetitive	19	1.1	0.22	1.93×10^{8}	0.35×10^{12}	1.8×10^{3}
Highly repetitive	9	-	-	-	0.16×10^{12}	-

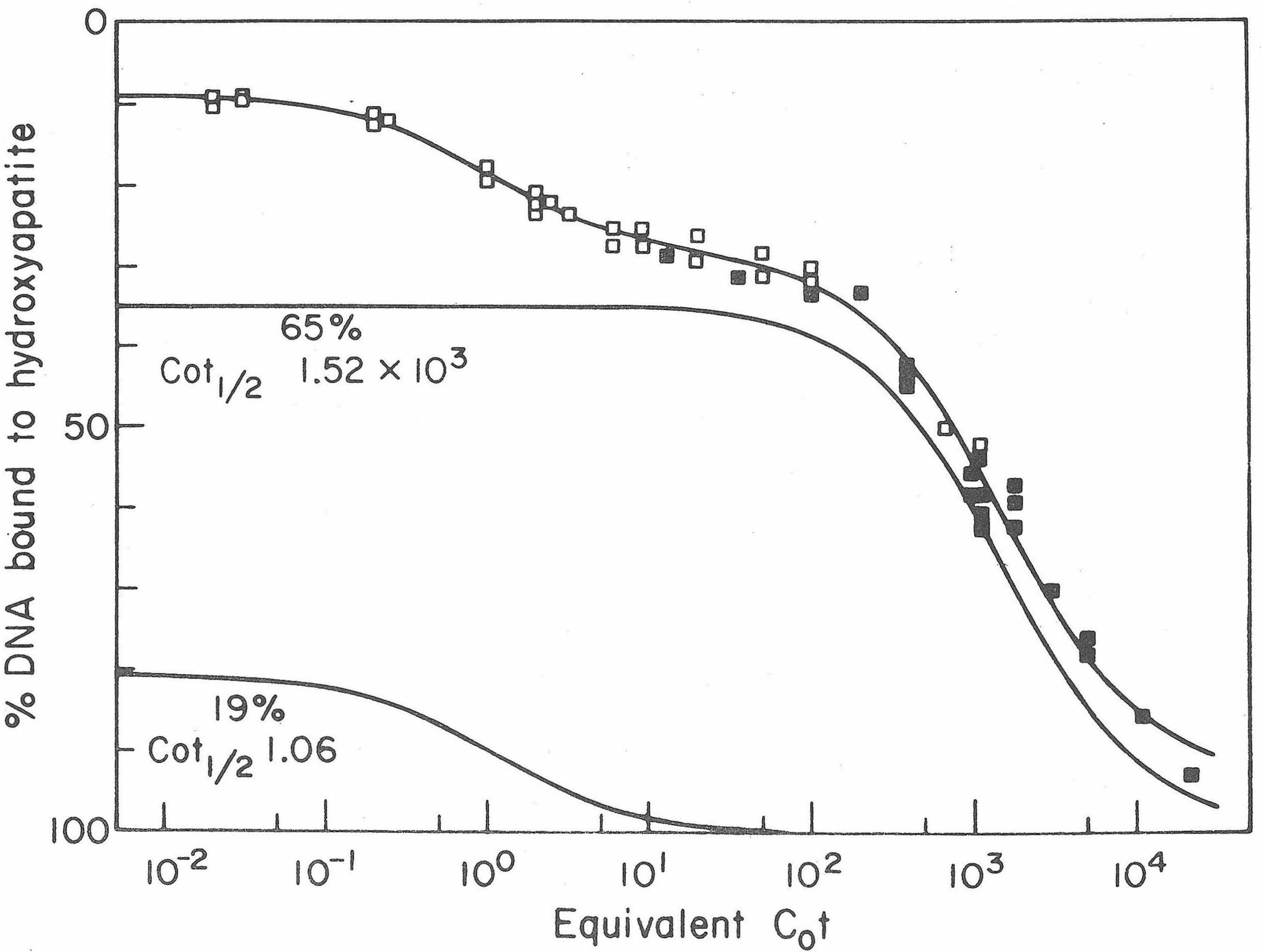
Calculated from the data of fig. 1

(a) The complexity of E. coli is assumed to be 2.8×10^{9} daltons (Cairns 1963). The effect of GC content on the rate of renaturation of rat DNA (41% GC) relative to E. coli DNA (50% GC) is to reduce the rate by 0.83 (Wetmur and Davidson, 1968) and assuming the GC content of each major reassociating class of rat DNA is the same.

(b) The rat haploid genome consists of 1.8×10^{12} daltons of DNA (Walker and Yates, 1952)

TABLE II: Parameters describing the hybridization of RNA to nuclear DNA present in excess

Class of RNA	% total RNA	Cot 1/2 observed
Slowly hybridizing HnRNA	37	1.7×10^3
More rapidly hybridizing HnRNA	12	5.0
rRNA	75	33.8



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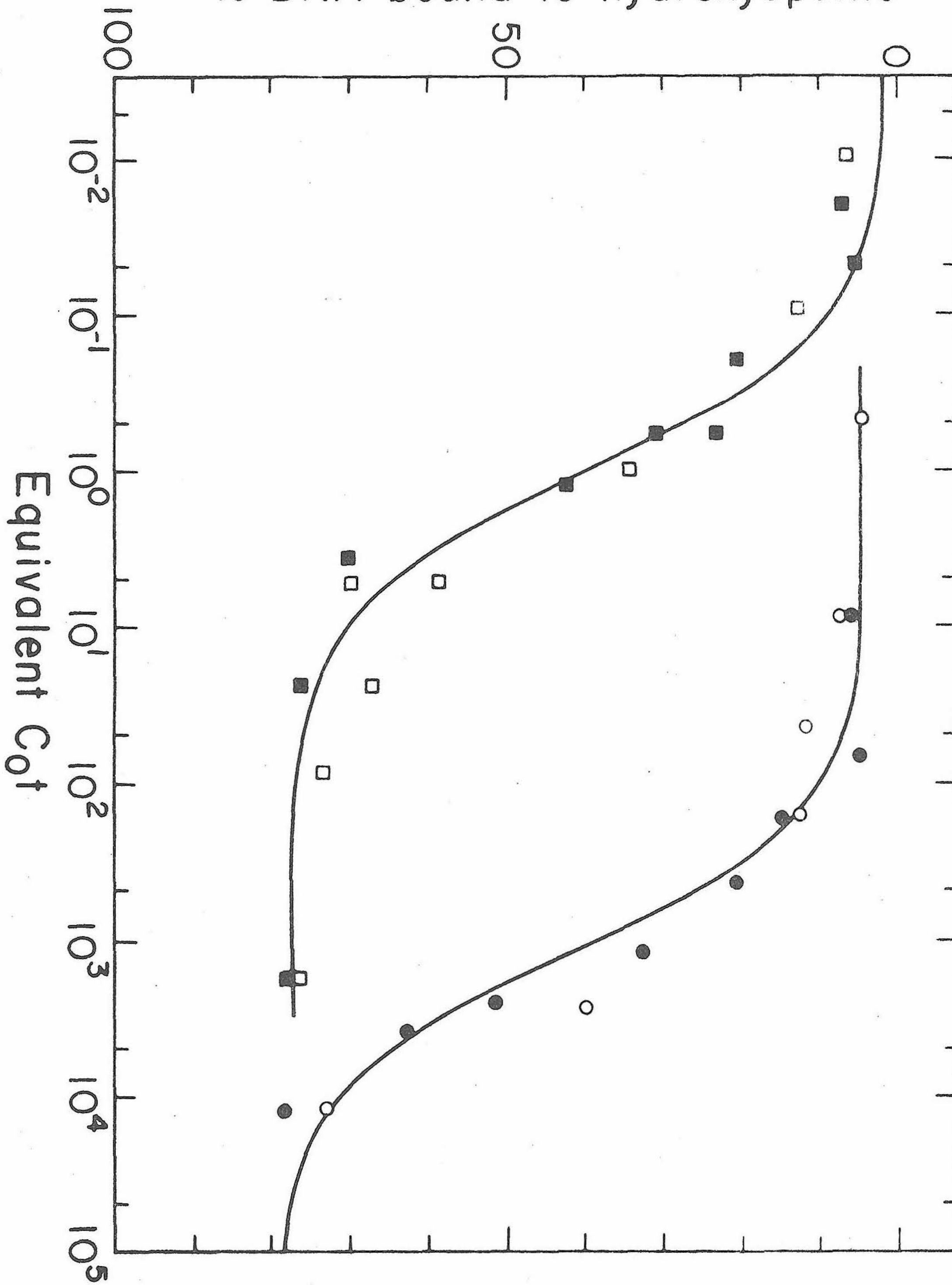

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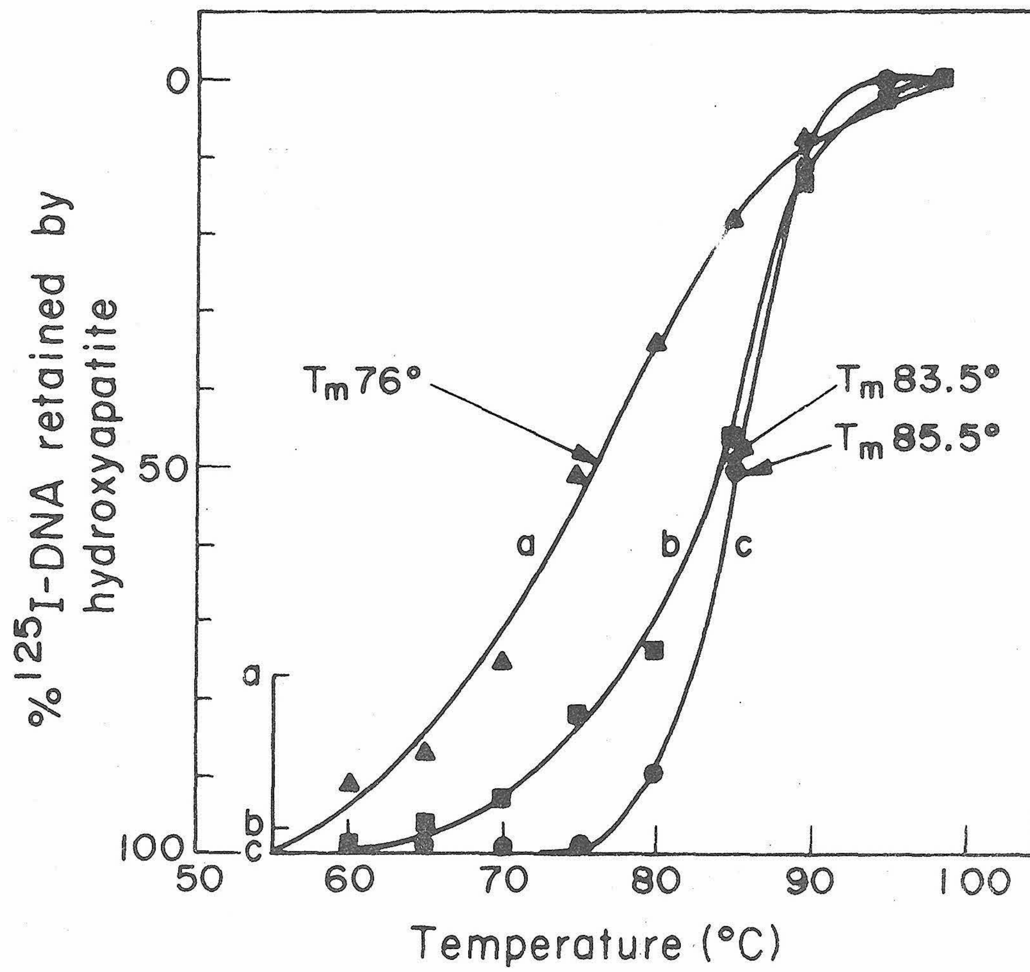

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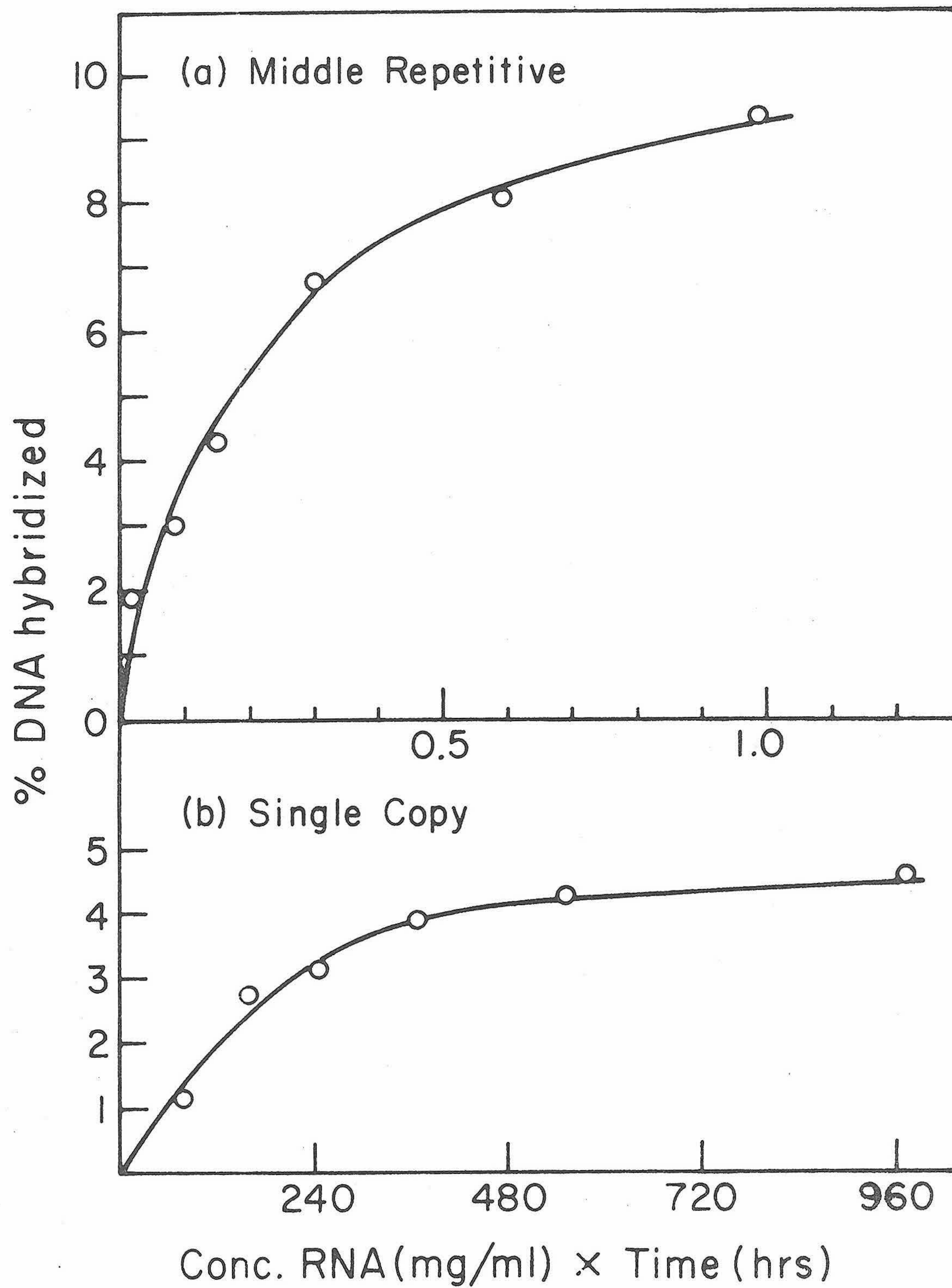

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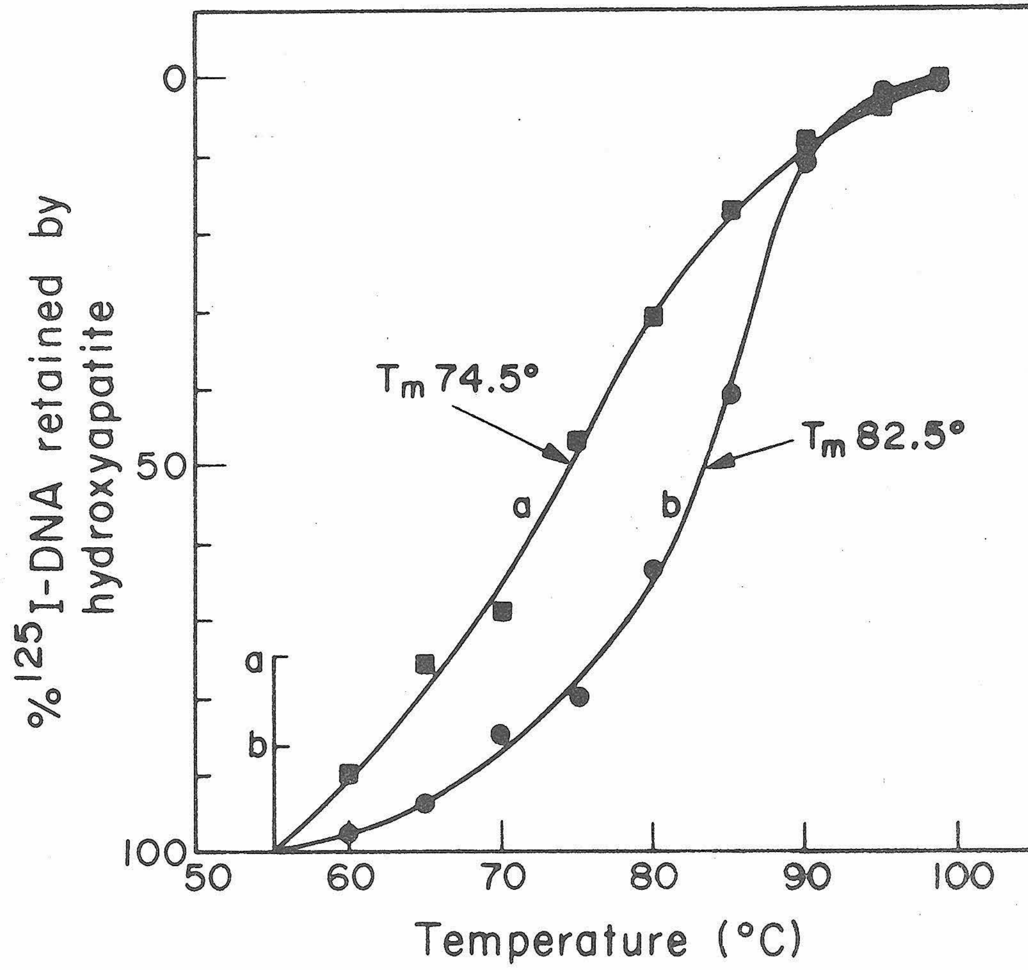

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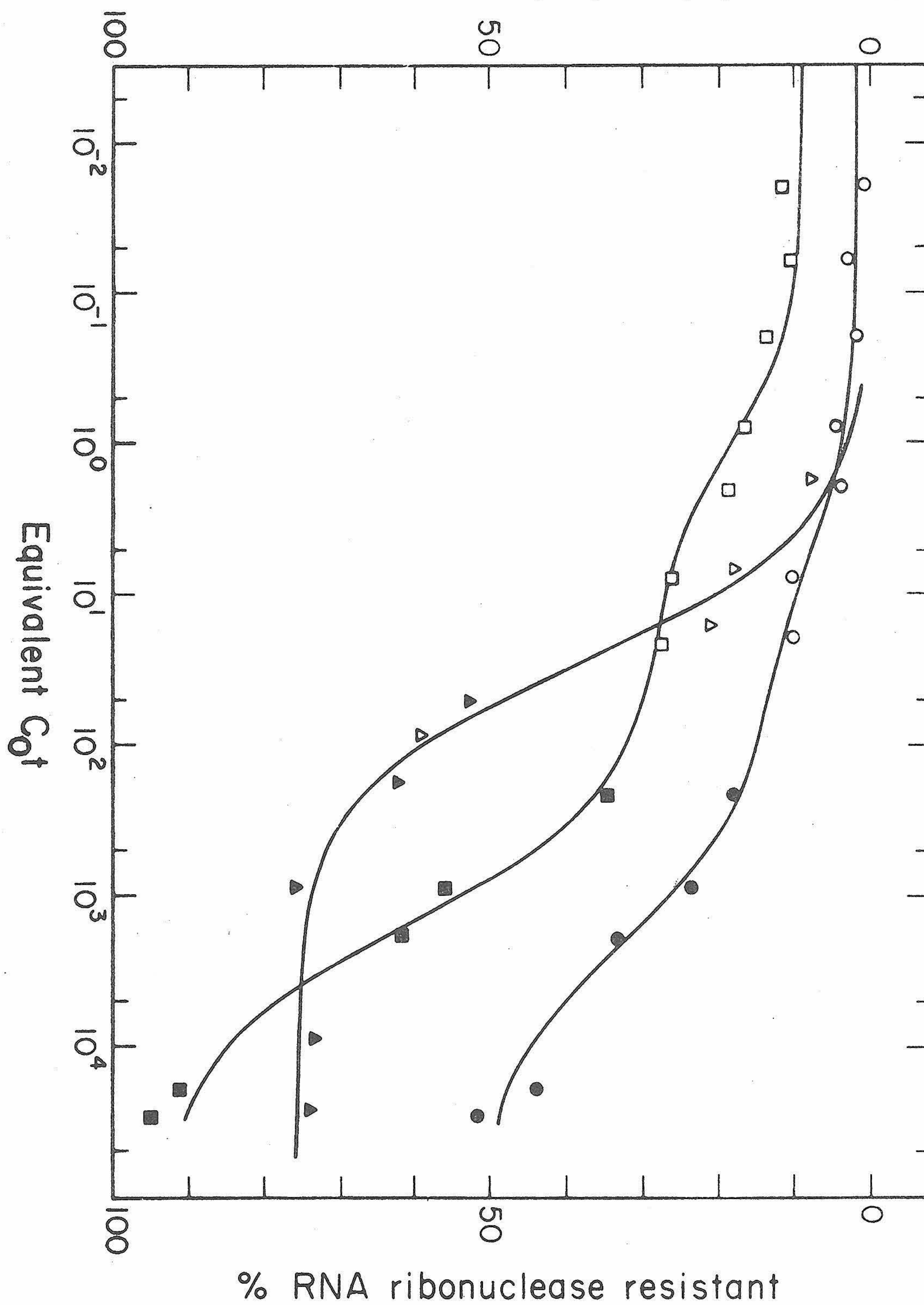

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CHAPTER 4

HYBRIDIZATION PROPERTIES OF CHROMOSOMAL RNA

ABSTRACT: A chromatin-associated RNA (cRNA) prepared from rat ascites cells hybridizes to about 16% of isolated middle repetitive and 1% of isolated single copy rat DNA. In a hybridization reaction to total DNA, present in excess, at least 50% of the cRNA hybridizes at an average rate similar to the major component of the middle repetitive DNA. These experiments indicate that the majority of cRNA consists of repetitive transcripts. Under conditions which assay essentially only repetitive transcripts cRNA hybridizes to about 4.7% and giant nuclear RNA (HnRNA) hybridizes to about 4.6% of total nuclear rat DNA immobilized on filters. The Tm of cRNA hybrids (73.5°) and HnRNA hybrids (75.5°) are considerably lower than the Tm of native rat DNA (85.5°). This lowering of Tm is probably attributable, at least in part, to base-pair mismatch. Under the same conditions of hybridization there is some hybridization competition for complementary DNA sites between cRNA and HnRNA, presumably between repetitive transcripts. Due to probable base-pair mismatch it is possible to infer only that there is a similarity between HnRNA and cRNA transcripts and not necessarily an identity.

INTRODUCTION

The preparation and properties of a class of RNA molecules (cRNA) associated with isolated chromosomes (chromatin) from a variety of tissues and organisms have been described earlier (cf. Holmes _et al._, 1972). cRNA is small (about 16,000 daltons), hybridizes to a large percentage of nuclear DNA (2-5%) and contains sequences not found in either tRNA or rRNA.

This paper demonstrates that cRNA from the chromatin of rat ascites cells consists predominantly of transcripts from the middle repetitive component of nuclear DNA. We have previously shown that giant nuclear RNA (HnRNA), with molecular weights of 5-10 x 10^6 daltons, contains both repetitive and single copy sequences (Holmes and Bonner, 1973b). The relationship between the repetitive sequences of HnRNA and cRNA is tested by hybridization competition. There is a similarity between some of these sequences at the level of sensitivity of these experiments. We discuss these results in the context of several possible models of precursor-product relationship between HnRNA and cRNA.

Methods

DNA and cRNA were prepared from rat Novikoff ascites chromatin by the method of Dahmus and McConnell (1969). HnRNA (5-10 x 10^6 daltons) was prepared from rat Novikoff ascites nuclei and sonicated to about 4-8S as described previously (Holmes and Bonner, 1973a,b). rRNA and tRNA were prepared from rat ascites cells as described previously (Holmes and Bonner, 1973a; Dahmus and McConnell, 1969).

Purified middle repetitive and single copy nuclear DNA were prepared as described elsewhere (Holmes and Bonner, 1973b). HnRNA, tRNA, rRNA and cRNA were labeled *in vitro* with ^{3}H-dimethyl sulfate by a modification of the procedure of Smith *et al*. (1968), as described elsewhere (Holmes and Bonner, 1973b). The specific activity of HnRNA was 76,000 cpm/μg and that of cRNA was 82,000 cpm/μg in our counting system. These specific activities correspond to about 3% of the total bases methylated. The specific activity of rRNA was 110,000 cpm/μg (4% bases methylated) and that of tRNA was 180,000 cpm/μg (6% bases methylated).

RNA hybridization to purified DNA components. ^{3}H -cRNA was hybridized to either purified middle repetitive or single copy nuclear DNA in phosphate buffer pH 6.8 — 1 mM EDTA as described elsewhere (Holmes and Bonner, 1973b). The reaction conditions are described in the legend to Fig. 3. Hybridization was assayed by chromatography on Sephadex G100 as described in the legend to Fig. 1.

cRNA-DNA hybrids eluted from Sephadex G100 were subjected to buoyant density centrifugation in CsCl as described by Hough and Davidson (1973).

RNA hybridization to excess total DNA. ^{3}H -cRNA was hybridized to excess total nuclear DNA [111,000:1 DNA to RNA ratio] sheared to about 350 bases (single strand length) as described elsewhere (Holmes and Bonner, 1973b). The reaction conditions are described in the legend to Fig. 4. Hybridization was monitored by the method of Melli *et al*. (1971).

DNA-RNA hybridization with DNA immobilized on filters. DNA was denatured at 100° for 10 min in 1/100 SSC and applied to nitrocellulose filters (Schleicher and Schuell B-6, 5 mm) in the presence of 6X SSC, as described by Gillespie and Spiegelman (1963). 12 μg or 2 μg of DNA containing trace amounts of ^{14}C -DNA were applied to the filters. 50-70% of the DNA remained on the filters at the end of the hybridization experiment under conditions given in the legend to Fig. 6.

Melting profile of cRNA-DNA and HnRNA-DNA hybrids. ^{3}H-cRNA or ^{3}H-HnRNA was hybridized to filters containing nuclear DNA, treated with RNase, washed and counted as described in the legend to Fig. 6. The filters were removed from the counting vials, dried for several hours at room temperature and washed on both sides with 1X SSC. The filters were heated from 55° to 98° at 5° intervals in 1 ml of 0.12 M phosphate buffer pH 6.8. A separate filter was used for each temperature increment. After heating it was washed on both sides with 0.12 M phosphate buffer pH 6.8, dried and counted in a toluene-based scintillation fluid in a Beckman 200-B scintillation system. The results were corrected for loss of DNA from the filter during heating.

Results

The DNA reassociation profile of sheared rat ascites nuclear DNA and the isolation of middle repetitive and single copy DNA is described elsewhere (Holmes and Bonner, 1973b). At the criterion chosen for reassociation [$(Na)^{+}$ = 0.18 at 62°, Tm - 23°] the single copy

DNA (Cot 1/2 = 1.52×10^3) comprises about 65% of the total DNA and middle repetitive DNA (Cot 1/2 = 1.06) makes up about 19% of the total DNA.

Hybridization of cRNA to middle repetitive and single copy DNA. Excess ^{3}H-cRNA is hybridized to either unlabeled middle repetitive or single copy rat DNA. At the end of the reaction the hybrids are subjected to mild ribonuclease treatment and passed over Sephadex G100 as shown in Fig. 1. The extent of hybridization is estimated from the radioactivity associated with the DNA in the excluded volume of the column. If DNA and ^{3}H-cRNA are mixed, treated with ribonuclease, and immediately passed over the column no radioactivity is found in the excluded volume. ^{3}H-cRNA is associated with the DNA in the excluded volume only after it is allowed to react under conditions permitting the formation of hybrids.

When an aliquot from the pooled, excluded fractions of the Sephadex G100 column is subjected to buoyant density centrifugation in CsCl, as shown in Fig. 2, all the detectable radioactivity is found between a density of 1.67 and 1.75 [density of native DNA = 1.694, denatured DNA = 1.7 and free RNA >1.9]. This supports the view that the ribonuclease resistant RNA is associated with DNA. Since the majority of cRNA bands in the region expected for single stranded or native DNA it is concluded that, on average, only one or two cRNA molecules (circa 50 nucleotides) are hybridized per DNA molecule (circa 350 nucleotides). Some formation of networks of DNA duplexes containing hybridized cRNA molecules may also occur.

Fig. 3 shows the extent of hybridization of ^{3}H-cRNA to purified middle repetitive and single copy DNA, using as a criterion for hybridization the appearance of ribonuclease resistant ^{3}H-cRNA in the void volume of Sephadex G100. About 16% of the middle repetitive and 1% of the single copy DNA are complementary to cRNA. The single copy DNA contains, in the limit, one copy of each family of the middle repetitive DNA. However hybridization of cRNA to these repetitive representations accounts for only about 0.2% of the observed hybridization to single copy DNA

$$\left[\frac{3/2500}{0.65} \times 10^2 \left(\frac{\text{\% total DNA hybridized by repetitive transcripts/no. copies per genome}}{\text{\% total DNA hybridized by single copy transcripts}} \times 10^2\right)\right]$$

A rough estimate of the repetitive transcript content of cRNA can be obtained from Fig. 3a. The half-reaction of hybridization of cRNA to middle repetitive DNA occurs at 0.007 mg/ml hr^{-1} (0.08 mol L sec^{-1}) yielding a pseudo first order rate constant of 10. It is estimated from the rate of formation of middle repetitive DNA duplexes that RNA hybridization to middle repetitive DNA should occur with a rate constant of about 30. Therefore it is estimated that 30-50% of cRNA consists of repetitive transcripts. By a similar calculation it is estimated that only 0.1% of the cRNA sequences are transcripts of single copy DNA. These estimates are not exact for reasons described previously (Holmes and Bonner, 1973b).

Several investigators have made use of hydroxyapatite to follow

the formation of DNA-RNA hybrids (Davidson and Hough, 1969; Gelderman et al., 1970; Brown and Church, 1971; Hahn and Laird, 1971; Firtel, 1972; Grouse et al., 1972; McConaughy and McCarthy, 1972; Holmes and Bonner, 1973b). This method can not be applied to the present work because cRNA-DNA hybrids do not bind quantitatively to hydroxyapatite under standard conditions. The reason for the failure of these hybrids to bind to hydroxyapatite is not known but could be due to the small size of the RNA-DNA duplexes. Hough and Davidson (1973) observed a similar inability of a portion of repetitive DNA-RNA hybrids from *Xenopus* to bind to hydroxyapatite although these hybrids met the criteria of duplex structures by Sephadex G200 chromatography and buoyant density in CsCl.

Hybridization of ^{3}H-cRNA to total nuclear DNA present in vast excess. ^{3}H-cRNA was hybridized to excess total nuclear DNA (sheared to about 350 bases, single strand length). The hybridization was carried out as described in the legend to Fig. 4. Under the conditions used the rate of hybridization is determined predominantly by the concentration of DNA sequences. The results of this experiment are shown in Fig. 4. The Cot 1/2 of the observed hybridization is 1.8. This is similar to the Cot 1/2 of the major reassociating component of middle repetitive DNA (1.06) but is different from that of rRNA (Cot 1/2 = 33.2) determined by the same procedure (Holmes and Bonner, 1973b).

The hybridization reaction goes to about 50% completion. Incomplete reaction is probably not due solely to degradation of RNA

resulting from the high temperature and long periods of incubation. Using a first order rate constant of 1.4×10^{-9} (Eigner et al., 1961) it is estimated that the weight-average molecular weight of cRNA is reduced from about 16,000 daltons to 13,000 daltons by thermal scission of phosphodiester bonds during the course of a hybridization reaction lasting 90 hours (Cot 1.2×10^{4}). Fig. 5 shows the pattern of chromatography on Sephadex G100 of cRNA reacted with DNA to a Cot of 2×10^{3} without subsequent ribonuclease treatment. About 15% of the radioactivity elutes in a position corresponding to nucleotides and small oligonucleotides confirming the low rate of breakdown of cRNA, and a further 10% of the radioactivity elutes in a position corresponding to free cRNA.Thus without ribonuclease treatment it is estimated that about 70-80% of the RNA is in hybrid form. An aliquot from the same reaction mix yields only 55% hybridization after ribonuclease treatment as judged by the TCA precipitation method. The difference in hybridization estimated by these two methods might be accounted for by the withdrawal of some of the cRNA into duplex structures which are ribonuclease sensitive. The formation of these duplex structures would prevent further hybridization of the RNA in these duplexes but, at the same time, the duplexes would not be scored as hybrids due to their ribonuclease sensitivity.

Hybridization of RNA to DNA immobilized on filters. Fig. 6 shows the results of hybridizing cRNA or HnRNA to DNA immobilized on filters (Gillespie and Spiegelman, 1967) under conditions that assay essentially only the hybridization of repetitive transcripts. HnRNA

and cRNA hybridize to approximately 4.6% and 4.7% of the total DNA respectively. Since about 19% of the total DNA is middle repetitive (Holmes and Bonner, 1973b) these values represent 24% and 25% of the middle repetitive DNA. This is somewhat above the values of 9.4% for HnRNA (Holmes and Bonner, 1973b) and 16% for cRNA (Fig. 3) obtained when the RNA is hybridized to purified middle repetitive DNA in solution.

The hybridization of tRNA and rRNA to DNA immobilized on filters has been included in Fig. 6 for comparison. Both the cRNA and HnRNA hybridize to about 100 times more DNA than rRNA and tRNA. Assuming a complexity of 2.45×10^{6} daltons for rRNA (Loening, 1968), 2.4×10^{4} daltons for tRNA (Tissières, 1959) and 1.8×10^{12} daltons for the rat haploid genome (Walker and Yates, 1952) it is estimated that there are approximately 130 and 7,400 genes for rRNA and tRNA respectively. These values are in good agreement with the findings of other workers (Brimacombe and Kirby, 1968; Mohan et al., 1969; and Quincey and Wilson, 1969).

The melting profiles of cRNA and HnRNA (hybridized to DNA on filters) are shown in Fig. 7. Both classes of hybrid have a lower Tm and a broader melting profile than do native DNA or single copy DNA-RNA hybrids (as judged by their thermal elution for hydroxyapatite (Holmes and Bonner, 1973b). It is not known to what extent the melting profiles of cRNA and HnRNA hybrids are attributable to the short size of the hybrids (Hayes et al., 1970), to base-pair mismatch, to base

composition effects or to methylation of the RNA bases (circa 3%) by in vitro reaction with ^{3}H dimethyl sulfate.

Hybridization competition between cRNA and HnRNA. The relationship between the repetitive transcripts of HnRNA and cRNA was tested by reciprocal competition hybridization as shown in Fig. 8. When cRNA is added to the reaction in sufficient quantities to nearly saturate all complementary DNA sites the extent of competition by simultaneously added unlabeled HnRNA is slight. This indicates that only a small fraction of the sequences present in HnRNA is complementary to cRNA. This is expected because only about 10-20% of the input HnRNA is represented by repetitive transcripts (Holmes and Bonner, 1973b). In the reciprocal competition, uniformly labeled HnRNA added at near saturating amounts is competed to an extent indicating that about 50% of the sequences of cRNA are found in HnRNA. Due to base-pair mismatch in the hybrids, as described above, it is possible to infer only that there is a similarity and not necessarily an identity between the competing repetitive sequences in cRNA and HnRNA.

Discussion

The experiments described in this and in two other papers in the present series (Holmes and Bonner, 1973a,b) provide evidence that: (i) a large portion (about 60% of the radioactivity in rat ascites RNA after a 30 min pulse of ^{3}H-uridine is associated with HnRNA molecules of 5-10 x 10^6 daltons as judged by sedimentation velocity

and electron microscopy under stringent denaturing conditions; (ii) this HnRNA contains both repetitive and single copy transcripts; (iii) a small RNA (cRNA) associated with chromatin (isolated chromosomes) consists predominantly of repetitive transcripts which have sequences in common with at least some of the repetitive sequences of HnRNA at the criterion chosen for testing this relationship.

It is improbable that contamination of HnRNA preparations with cRNA can explain the competition experiments because HnRNA consists of molecules in the weight range 5-10 x 10^6 daltons (Holmes and Bonner, 1973a) while cRNA has a molecular weight of about 16,000 daltons (Dahmus and McConnell, 1969). However, serious consideration should be given to the possibility that cRNA is an artifactual degradation product of HnRNA. It is unlikely that cRNA consists of random breakdown products of total HnRNA because at least 50% of cRNA consists of repetitive transcripts, whereas HnRNA consists mainly of single copy transcripts (Holmes and Bonner, 1973b). However it is possible that cRNA is the breakdown product of either (i) a portion of HnRNA which might be organized predominantly as contiguous repeated sequences or (ii) repeated sequences of HnRNA which are interspersed between single copy sequences. We will return to this point presently.

The observations that rat ascites cRNA becomes labeled considerably later than HnRNA after a short labeling pulse and that new sequences of cRNA occur some time after the production of new sequences of repetitive HnRNA in rat liver (Mayfield & Bonner, 1972) are consistent with

the idea that cRNA is not a breakdown product of HnRNA resulting from some isolation procedure. However if cRNA is a "biological" product formed from the repetitive segments of HnRNA, the possibility cannot be ruled out that it undergoes further degradation during isolation. It is unlikely that cRNA from rat ascites cells is degraded tRNA or rRNA although this possibility cannot be ruled out for preparations from all tissues (Dahmus and McConnell, 1969; Holmes et al., 1972).

Two experiments provide circumstantial evidence that cRNA is correlated with gene activity. New sequences of cRNA are produced during rat liver regeneration (Mayfield and Bonner, 1972) and different sequences of cRNA are found in different tissues of the same organism (Bonner and Widholm, 1967 ; Mayfield and Bonner, 1971). An analogy may be drawn between cRNA and activator RNA in the model of gene regulation proposed by Britten and Davidson (1969). In this model activator RNA is able to recognize specific DNA sequences, termed receptor genes, which are adjacent to batteries of producer genes. The interaction of activator RNA with a receptor gene controls the activity of the associated battery. The Britten-Davidson model calls for considerable repetition of receptor genes and consequently of the activator RNA as well. cRNA fulfills this expectation by virtue of its hybridization to middle repetitive DNA. In the Britten-Davidson model activator RNAs are transcribed from integrator genes whose transcription is controlled by associated sensor genes. The interaction of stimulating agents, such as hormone-protein complexes, with sensor genes, results in the activation of associated

integrator genes. Since cRNA may be produced by the specific cleavage of some of the repetitive sequences of HnRNA it is conceivable that a portion of HnRNA may contain transcripts of integrator sets. In this way a stimulus such as that evoked by partial hepatectomy of the liver could result in the production of polycistronic RNA transcripts from integrator sets which is post-transcriptionally cleaved to yield activator RNAs.

Alternatively HnRNA could be the polycistronic product of producer genes. The presence of a small proportion of repetitive sequences in HnRNA might reflect the transcription of operator or RNA polymerase binding sites or perhaps sequences involved in the processing and transport of HnRNA (Georgiev *et al.*, 1972). In these cases cRNA could constitute by-products of the post-transcriptional modification of HnRNA.

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Figure Legends

Fig. 1. Chromatography of cRNA-DNA hybrids on Sephadex G100. ^{3}H-cRNA was hybridized to unlabeled middle repetitive DNA (45 mg/ml x hr) and treated with ribonuclease as described in the legend to Fig. 3. The mixture was then passed over Sephadex G100. Dextran 2000, isolated cRNA and a mixture of 2'-3'-ribonucleotides (Calbiochem) were used to standardize the column (2 x 45 cm). The column was eluted with 0.12 M phosphate buffer pH 6.8 - 1 mM EDTA at 4°. 2.5 ml fractions were collected.

Fig. 2. Buoyant density centrifugation in CsCl of ^{3}H-cRNA hybrids. The three peak fractions of the excluded volume peak of Sephadex G100 (see Fig. 1) were pooled and 3 ml added to 4 g of CsCl. The samples were overlaid with oil and centrifuged at 25° for 52 hr at 32,500 rev/min in a Spinco 50.1 swinging bucket rotor. At the end of centrifugation the tubes were dripped onto Whatmann 3 MM filters, washed with 10% TCA, dried and counted. The refractive index of some of the fractions was measured to monitor the CsCl gradient.

Fig. 3. Hybridization of dimethyl sulfate labeled ^{3}H-cRNA (82,000 cpm/μg) from rat ascites to purified kinetic components of rat nuclear DNA (sheared to about 350 bases single strand length). After hybridization the reaction mixture was adjusted to 0.24 M phosphate buffer pH 6.8 and treated with ribonuclease A (50 μg/ml) and ribonuclease T1 (50 units/ml) at 37° for 10° and chromatographed on

Sephadex G100 (see Fig. 1). The ^3H-cRNA which eluted in the void volume of Sephadex G100 was scored as hybrid.

(a) Hybridization to middle repetitive DNA. Reaction carried out in 0.12 M phosphate buffer pH 6.8 - 1 mM EDTA at 62°. Ratio of RNA to DNA about 440:1.

Concentration of RNA (mg/ml)	Hours Incubation	% Hybridization
0.001	0.25	2.7
0.001	0.25	2.5
0.001	0.5	6.1
0.001	1.15	10.4
0.001	2.55	11.5
0.001	2.55	12.3
0.001	11.5	16.3
0.001	11.5	17.3
0.91	9.75	16.6
0.91	9.75	15.4
9.2	5.0	16.2
9.2	5.0	16.8

(b) Hybridization to single copy DNA. Reaction carried out in 0.48 M phosphate buffer pH 6.8 - 1 mM EDTA at 66°. Ratio of RNA to DNA about 210:1.

Concentration of RNA (mg/ml)	Hours Incubation	% Hybridization
21	1.8	0.23
21	4.0	0.44
21	7.2	0.6
21	11.0	0.84
21	18.0	1.04

Fig. 4. Computer analysis of the hybridization of dimethyl sulfate labeled ^3H-cRNA (82,000 cpm/μg) from rat ascites in the presence of an excess of rat nuclear DNA (350 nucleotides single strand length). The ratio of ^3H-cRNA to DNA was about 1:111,000.

The hybridization was carried out in either (open symbols) 0.12 M phosphate buffer pH 6.8 - 1 mM EDTA or (filled symbols) 0.48 M phosphate buffer pH 6.8 - 1 mM EDTA at 62° or 66° respectively. The reassociation of DNA (squares) was followed by monitoring the A260 of an aliquot of the reaction mixture after passage through a hydroxyapatite column (Britten and Kohne, 1967). After mild ribonuclease treatment (20 μg/ml ribonuclease A and 20 units ribonuclease T1 in 0.24 M phosphate buffer for 15 min at 37°) the RNA:DNA hybrids (circles) were monitored by the TCA precipitation method of Melli et al. (1971).

Fig. 5. Chromatography on Sephadex G100 of ^{3}H-cRNA-DNA hybrids (incubated to a Cot of 2 x 10^2 as described in the legend to Fig. 4). The hybrids were not subjected to ribonuclease treatment. Chromatography carried out as described in the legend to Fig. 1.

o——o ^{3}H cpm x 10^{-2} ●——● ^{3}H cpm x 10^{-4}

Fig. 6. Hybridization of ^{3}H-RNA to total rat DNA immobilized on filters. Hybridization was carried out at 37° for (a) 18 hr in the presence of 0.12 M sodium phosphate buffer pH 6.8 and 50% (v/v) formamide or (b) 24 hr in 5 x SSC (0.15 M sodium chloride - 0.015 M sodium citrate) and 50% (v/v) formamide in a final volume of 0.2 ml. Each vial contained 2 DNA filters and one blank filter. Before the addition of the filters the hybridization solution containing the RNA was heated to 100° for 5 min and cooled rapidly in ice. Following incubation, filters were rinsed in 2X SSC, washed by filtration on

both sides with 2X SSC and treated with a mixture of 50 µg/ml preboiled pancreatic RNase A and 50 units/ml T1 RNase at 37° for 0.5 hr in 2X SSC. The filters were again rinsed in 2X SSC and washed by filtration in 2X SSC, dried and counted.

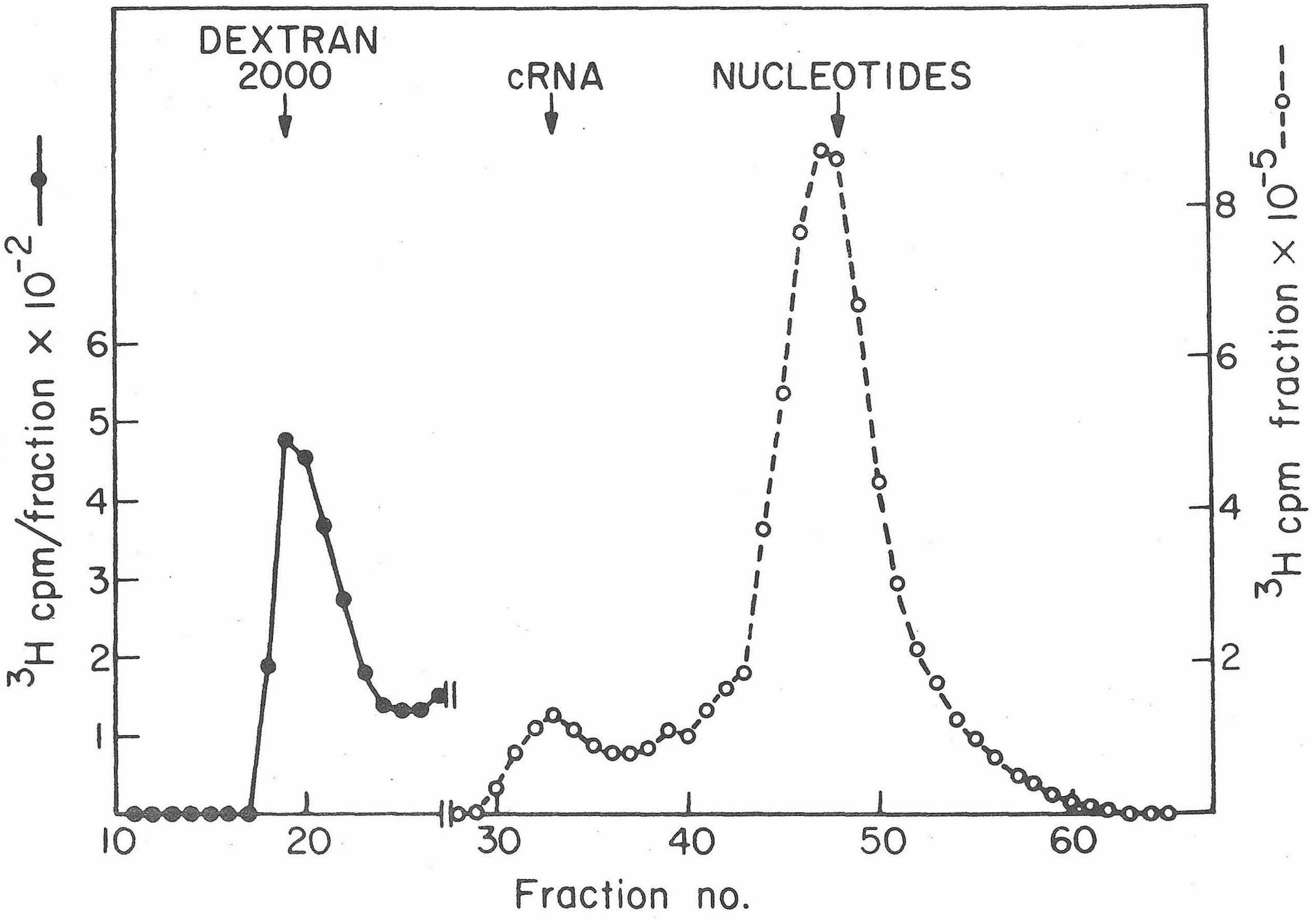
(a) ●—● ^{3}H-cRNA (82,000 cpm/µg); O—O ^{3}H-HnRNA (76,000 cpm/µg), 10 µg input DNA per filter. (b) O—O ^{3}H-tRNA (180,000 cpm/µg); ●—● ^{3}H-rRNA (110,000 cpm/µg). 2 µg input DNA per filter.

Fig. 7. Integral melting profile of ●—● ^{3}H-HnRNA and ■—■ ^{3}H-cRNA after hybridization to filters containing DNA (as described in legend to Fig. 6). Melting profile prepared as described in Methods. The bars at left on the figure indicate ^{3}H-RNA melting at 60°.

Fig. 8. Hybridization competition between ^{3}H-cRNA (82,000 cpm/µg) and ^{3}H-HnRNA (76,000 cpm/µg) (sonicated to about 4-8S in size). Hybridization competition carried out by simultaneous addition of labeled and unlabeled RNA to DNA (containing trace amounts of ^{14}C-DNA) immobilized on filters as described in the legend to Fig. 6. Percent hybridization estimated as described in legend to Fig. 6.

●—● ^{3}H-cRNA x unlabeled HnRNA

O—O ^{3}H-HnRNA x unlabeled cRNA



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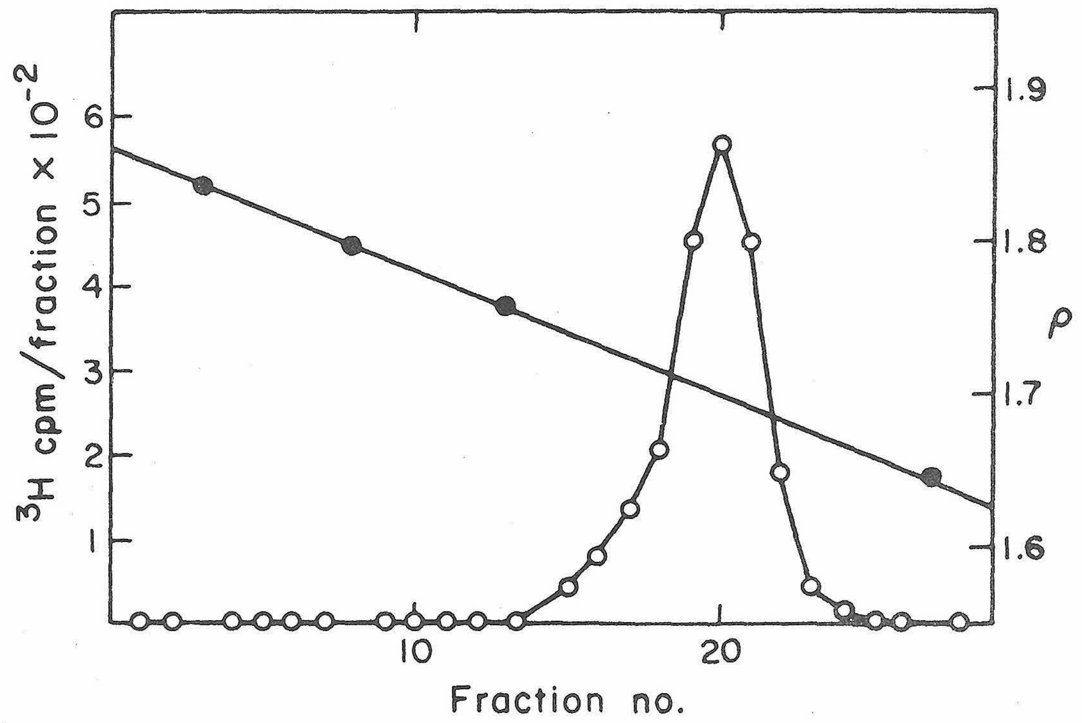

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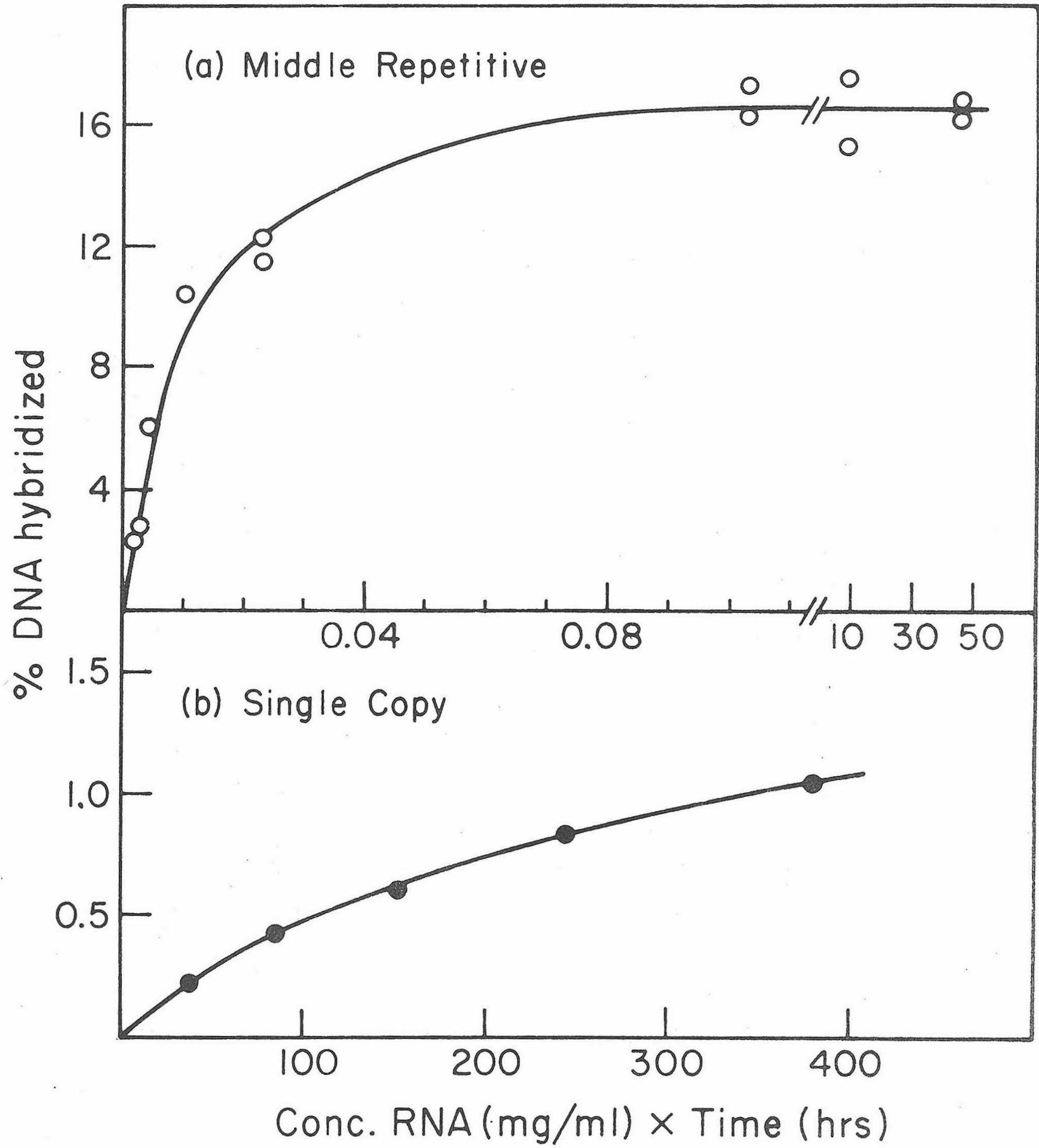

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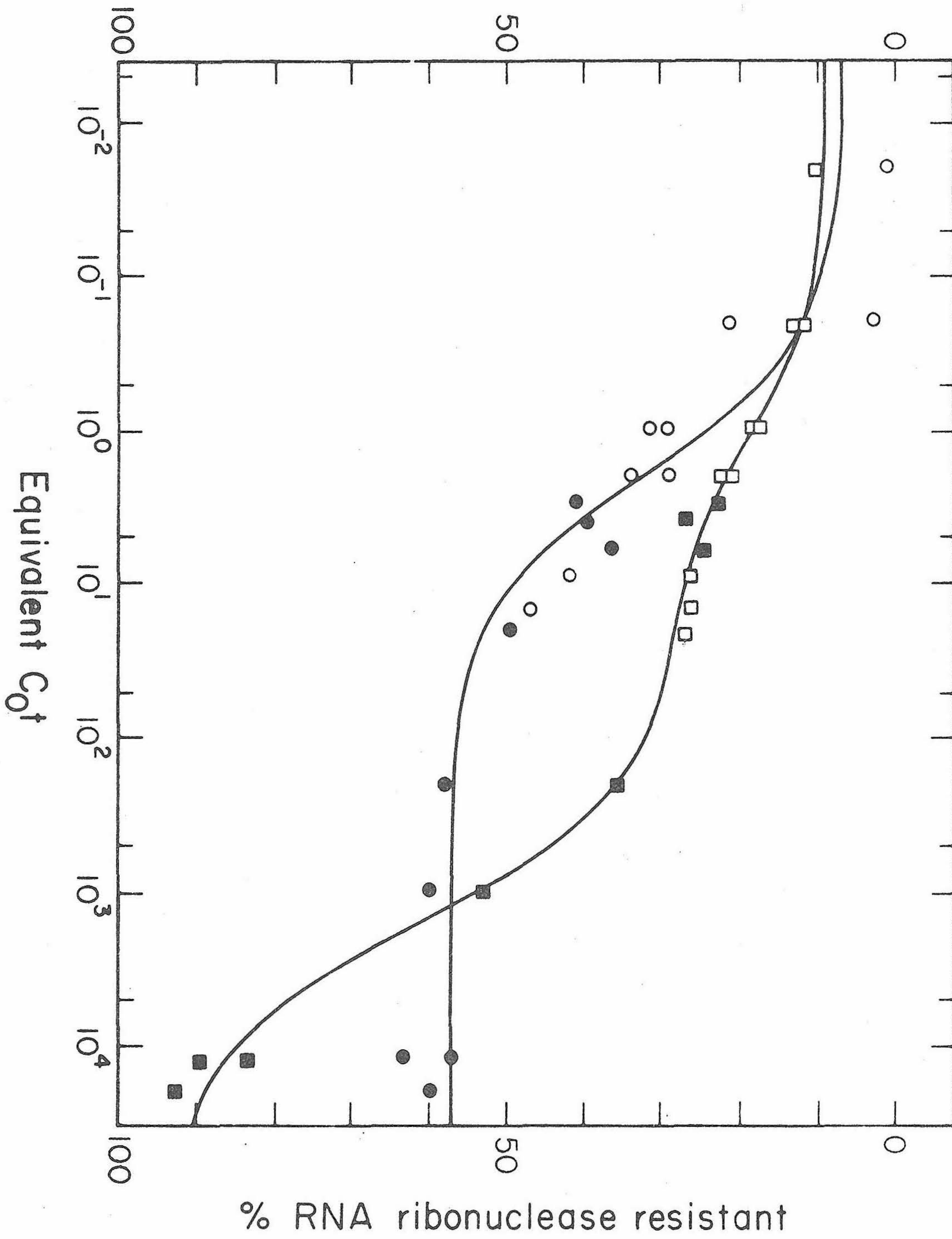

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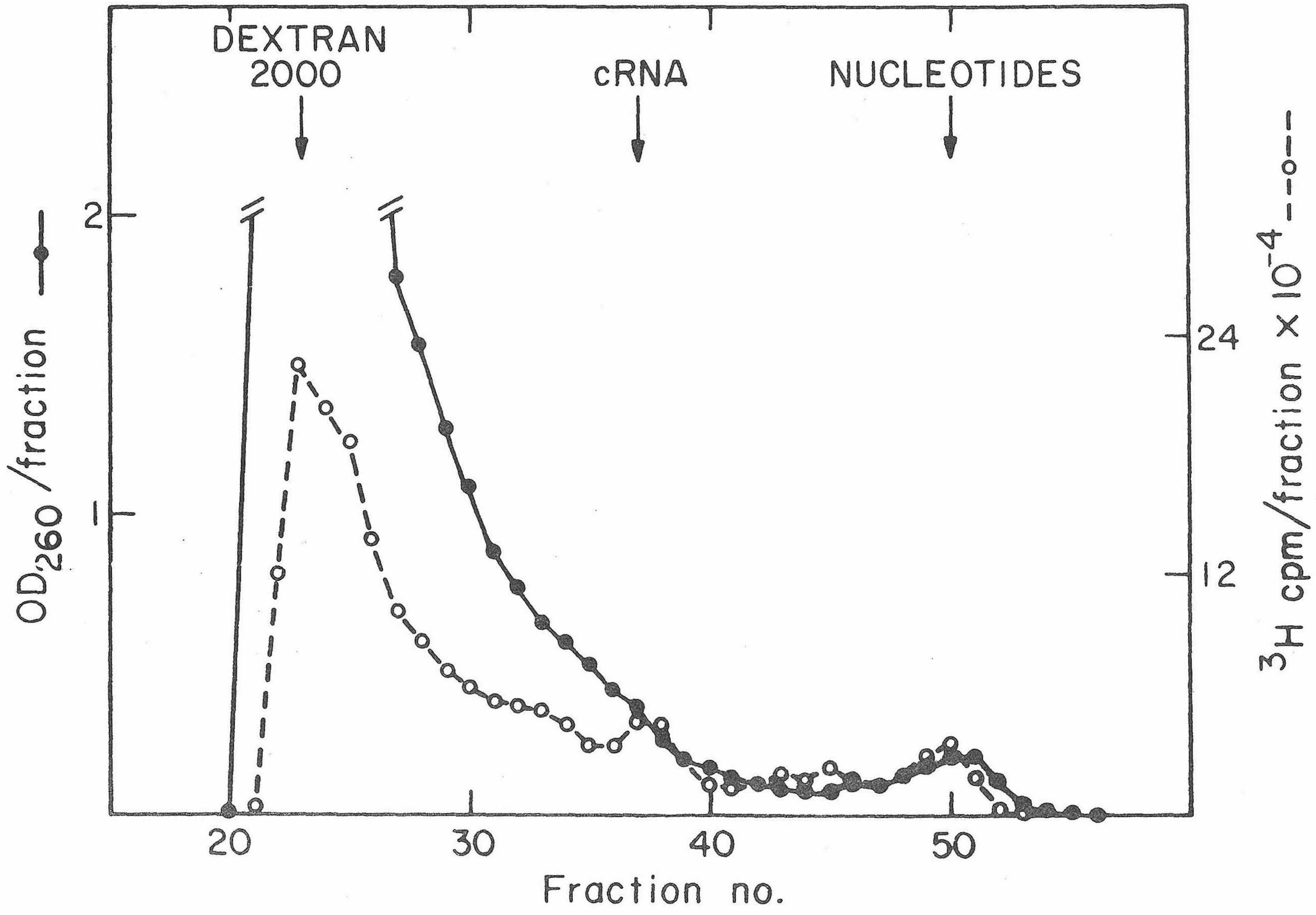

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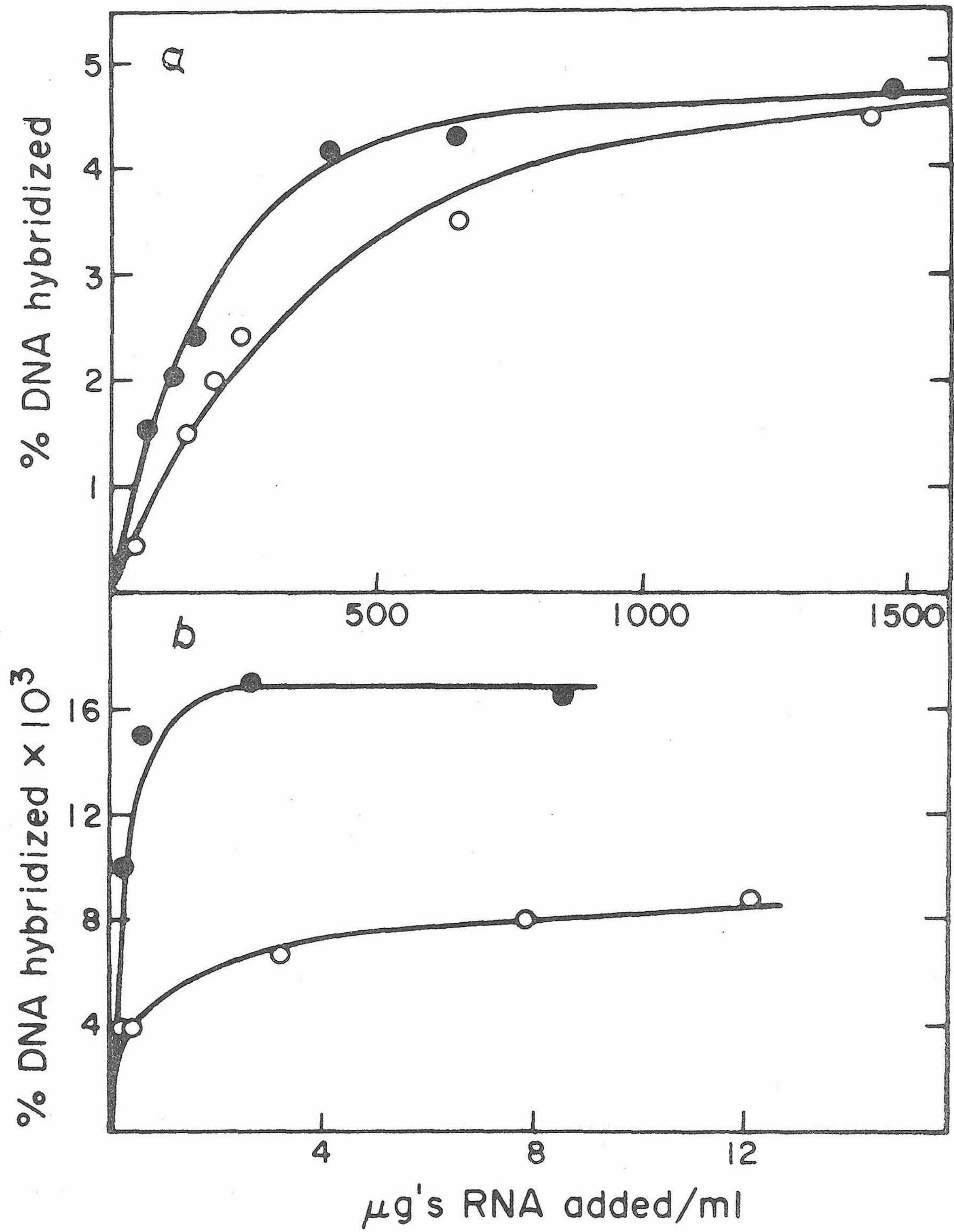

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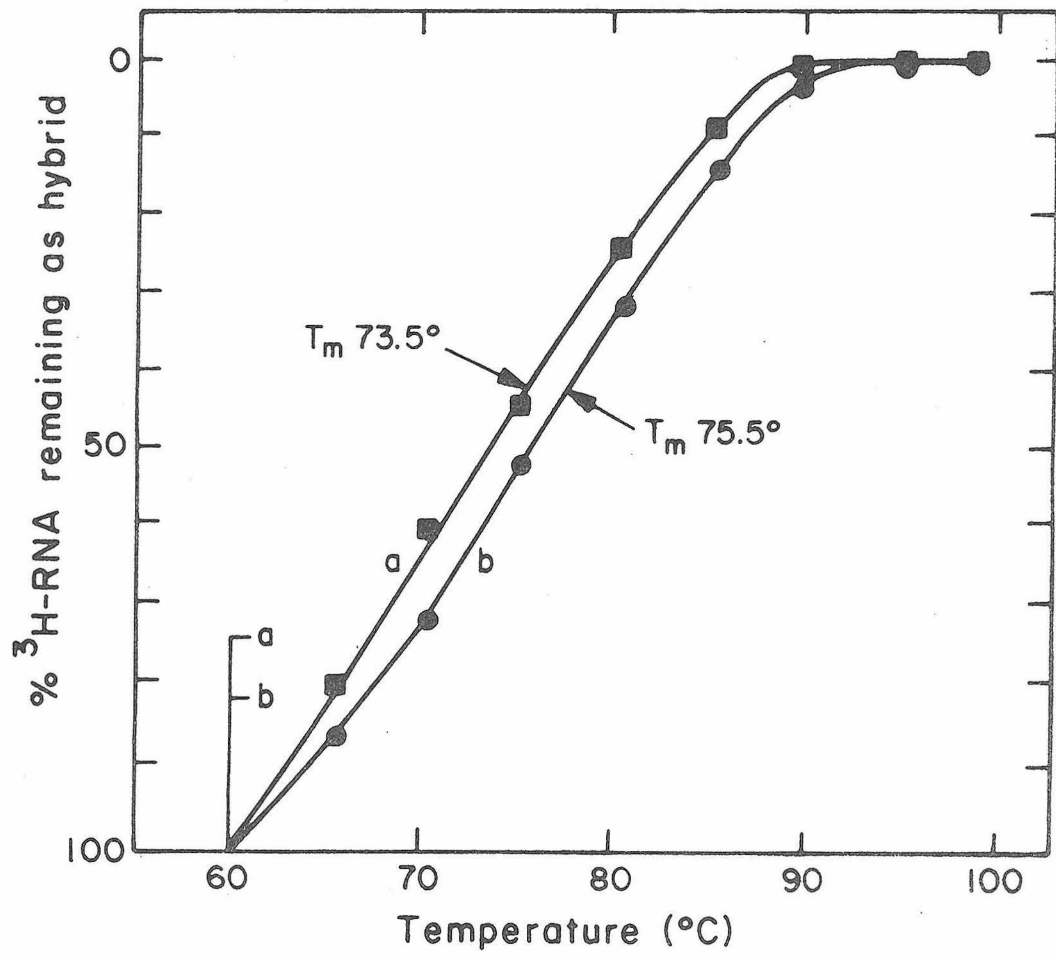

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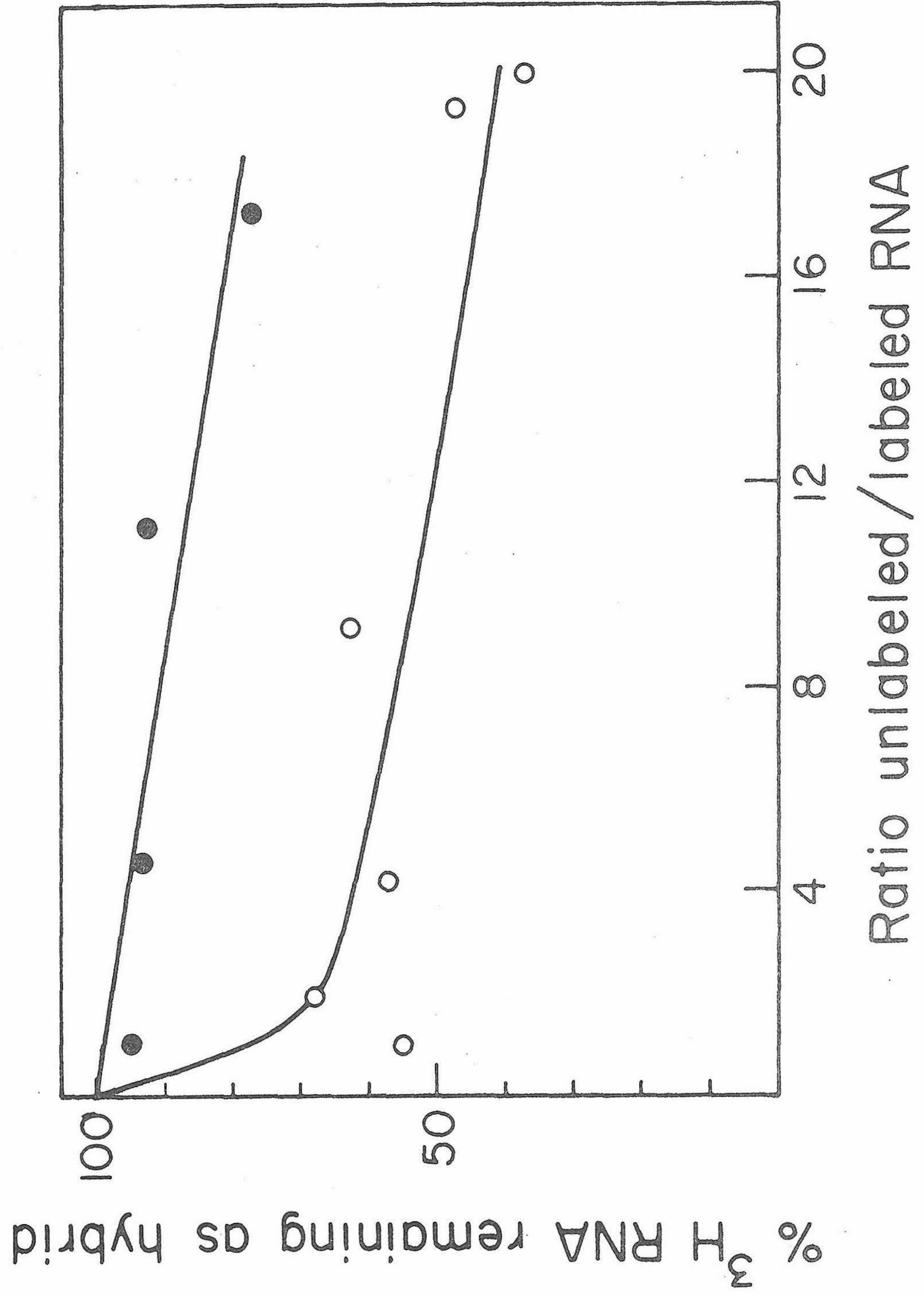

(B)